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THE EFFECT OF TESTICLE EXTRACT ON RED BLOOD CELLS IN VITRO

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(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, April 14, 1931)

The experiments of Duran-Reynals (1, 2) demonstrating that testicle extract, and, to a less degree, the extracts of certain other organs (kidney, skin, etc.) are endowed with the property of enhancing the lesions produced by vaccine virus and staphylococcus have been confirmed by McClean (3), Maitland (4) and others. Hoffman (5) and Pijoan (6) extended these results to many filtrable viruses and bacteria. The action of testicle extract is not clearly understood, although, since the first experiments of Duran-Reynals, it has appeared that the action is on the tissues of the animals and not directly on the vaccine virus itself. Duran-Reynals (1) has demonstrated that vaccine virus, when injected intravenously, localizes very readily in areas of skin previously injected with testicle extract. McClean (3) and Hoffman and Duran-Reynals (7) have shown that testicle extract increases the spread of inert substances (India ink) injected intradermally, probably by increasing the permeability of tissues and cells.

In the present paper some experiments are reported which were undertaken to test the view that the action of the effective factor in testicle extract is upon the host tissue. Since it would have been difficult to demonstrate directly any modification of the permeability of tissues into which the extract had been injected, an indirect method, allowing it to act on cells *in vitro*, has been chosen. Red blood cells were used for these experiments, since it is possible to observe very easily a modification of the permeability of their membranes by means of the so called "fragility test."

Some preliminary tests were made by mixing rat testicle extract and rabbit red blood cells, washing the latter and then adding them to graded solutions of sodium chloride. It was found that the fragility of the cells was considerably increased. Later a series of experiments was carried out, in which the action of testicle extracts on red blood cells of various species was tested.

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* Fellow of the Rockefeller Foundation.

Method

All experiments were carried out according to the following technique:

Testicle and spleen extracts from rat, rabbit and guinea pig were prepared by grinding the organs with sand in a mortar, and adding an equal weight of 0.9 per cent salt solution. The resulting pulp was immediately centrifuged and only the supernatant fluid was used. Red cell suspensions were prepared as usual by centrifuging the defibrinated blood, aspirating off the serum and washing the cells three or four times in normal saline solution. After the last centrifugation, the preparations were brought to the original volume by the addition of normal saline solution. Both extract and red blood cells were used immediately, or at latest on the following day after remaining overnight in the ice box.

Each experiment was performed by mixing 1 cc. of red cell suspension with 1 cc. of testicle extract. The mixture was incubated for 2 or 3 hours at 37°. As control a mixture of red cell suspension and normal saline solution was kept for the same length of time in the incubator. The mixtures were centrifuged and the supernatant fluid aspirated off. The red cells were then washed with saline solution and, after centrifugation, brought to 2 cc. with it. A drop of the suspension was added to 1 cc. of saline solution at various concentrations, 0.90 per cent, 0.85 per cent, 0.80 per cent and so on down to 0.35 per cent. The control material was treated in the same way.

The amount of hemolysis of the red cells in each of the tubes was recorded, after they had been kept for about 30 minutes at room temperature and overnight in the ice box.

Experiments with Testicle Extract

In the first set of experiments, testicle extracts from rat, rabbit and guinea pig were tested against the red cells of each of these three species. The results with rat testicle extract are shown in Table I.

Table II shows the result of the experiments with rabbit testicle extract.

The results of the experiments with guinea pig testicle extract are shown in Table III.

Tables I, II and III show that testicle extract considerably increases the fragility of red cells. The cells of each species are not, however, sensitive in the same degree to the action of testicle extract.

These tests have been repeated several times, and the same results have always been obtained. It will be noted from these tables that the rat cells are little affected by any of the testicle extracts,

TABLE I

Hemolysis Induced by Rat Testicle Extract

Salt concentration, *per cent*	0.90	0.85	0.80	0.75	0.70	0.65	0.60	0.55	0.50	0.45	0.40	0.35
Red cells of rat + T. E.	+	+	++	++	++	++	++	++	++	++++	++++	++++
Control	–	–	–	–	–	–	–	–	–	+	++	+++
Red cells of rabbit + T. E.	+++	+++	+++	+++	+++	+++	+++	+++	+++	++++	++++	++++
Control	–	–	–	–	–	–	–	–	±	+++	++++	++++
Red cells of guinea pig + T. E.	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Control	–	–	–	–	–	–	–	–	–	+	++	+++

TABLE II

Hemolysis Induced by Rabbit Testicle Extract

Salt concentration, *per cent*	0.90	0.85	0.80	0.75	0.70	0.65	0.60	0.55	0.50	0.45	0.40	0.35
Red cells of rat + T. E.	–	–	–	–	–	–	++	++	++	++++	++++	++++
Control	–	–	–	–	–	–	–	–	+	+++	+++	++++
Red cells of rabbit + T. E.	–	–	–	–	–	–	+	++	+++	+++	++++	++++
Control	–	–	–	–	–	–	–	+	+++	++++	++++	++++
Red cells of guinea pig + T. E.	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Control	–	–	–	–	–	–	–	–	–	±	++	+++

TABLE III

Hemolysis Induced by Guinea Pig Testicle Extract

Salt concentration, *per cent*	0.90	0.85	0.80	0.75	0.70	0.65	0.60	0.55	0.50	0.45	0.40	0.35
Red cells of rat + T. E.	–	–	–	–	–	–	–	–	+	+++	++++	++++
Control	–	–	–	–	–	–	–	–	+	++	+++	+++
Red cells of rabbit + T. E.	–	–	+	+	++	++	++	+++	+++	+++	+++	++++
Control	–	–	–	–	–	–	–	+	++	+++	++++	++++
Red cells of guinea pig + T. E.	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Control	–	–	–	–	–	–	–	–	–	–	+	+++

whereas guinea pig cells are greatly affected by all. Rat testicle extract is the most active in increasing fragility, and guinea pig extract the least active. In other words, within the limits of our observations, the species giving the most active testicle extract has the least sensitive red cells. The following table summarizes the action of the various testicle extracts against the several kinds of red cells.

TABLE IV

Testicle extract	Red blood cells		
	Rat	Rabbit	Guinea pig
Rat	±	++	++++
Rabbit	±	+	+++
Guinea pig	–	+	++

TABLE V

Hemolysis Induced by Rat Spleen Extract

Salt concentration, *per cent*	0.90	0.85	0.80	0.75	0.70	0.65	0.60	0.55	0.50	0.45	0.40	0.35
Red cells of rat + S. E.	–	–	–	–	–	–	+	++	++	+++	++++	++++
Control	–	–	–	–	–	–	–	–	–	++	+++	++++
Red cells of rabbit + S. E.	–	–	–	–	–	–	–	+++	++++	++++	++++	++++
Control	–	–	–	–	–	–	–	±	+++	++++	++++	++++
Red cells of guinea pig + S. E.	–	–	–	–	–	–	–	–	–	++	+++	++++
Control	–	–	–	–	–	–	–	–	–	+	+	+++

The action of rat testicle extract against guinea pig red cells is so intense that a direct hemolysis of the cells has consistently been found after incubation. Such hemolysis has only occasionally been observed, and then only in a minor degree, in the case of mixtures of rabbit or guinea pig testicle extract with guinea pig red blood cells.

Experiments with Spleen Extract

The experiments of Duran-Reynals (1) have shown that extracts of several mesenchymatous organs and tissues yield results the opposite of those with extracts of testicle. Instead of enhancing, they either do not modify or they inhibit infection. Spleen extract was notable in this respect, and hence the effect of spleen extract on red cells has

TABLE VI

Hemolysis Induced by Rabbit Spleen Extract

Salt concentration, *per cent*.........	0.90	0.85	0.80	0.75	0.70	0.65	0.60	0.55	0.50	0.45	0.40	0.35
Red cells of rat + S. E.	–	–	–	–	–	–	–	–	–	+++	++++	++++
Control	–	–	–	–	–	–	–	–	–	++	+++	++++
Red cells of rabbit + S. E.	–	–	–	–	–	–	–	–	+++	++++	++++	++++
Control	–	–	–	–	–	–	–	–	+	++++	++++	++++
Red cells of guinea pig + S. E.	–	–	–	–	–	–	–	–	–	++	+++	+++
Control	–	–	–	–	–	–	–	–	–	±	+++	+++

TABLE VII

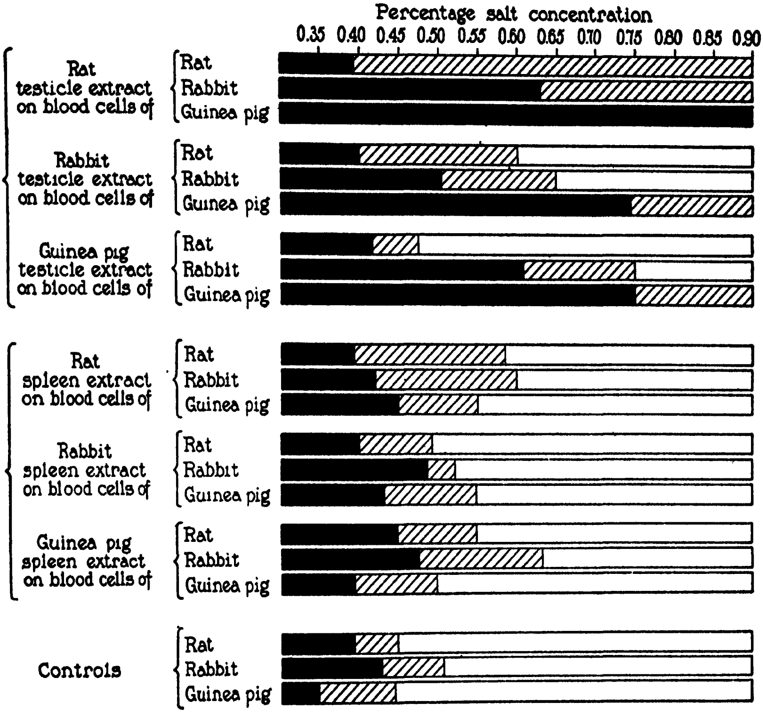
Hemolysis Induced by Guinea Pig Spleen Extract

Salt concentration, *per cent*........	0.90	0.85	0.80	0.75	0.70	0.65	0.60	0.55	0.50	0.45	0.40	0.35
Red cells of rat + S. E.	–	–	–	–	–	–	–	–	++	+++	++++	++++
Control	–	–	–	–	–	–	–	–	+	++	+++	+++
Red cells of rabbit + S. E.	–	–	–	–	–	–	+	+	+	++++	++++	++++
Control	–	–	–	–	–	–	–	+	+	+++	++++	++++
Red cells of guinea pig + S. E.	–	–	–	–	–	–	–	–	–	+	+++	++++
Control	–	–	–	–	–	–	–	–	–	–	+	+++

special interest. Several experiments have been carried out by the same technique used in the testicle extract experiments.

Tables V, VI and VII show the results of the first series of experiments with spleen extracts.

The results of these experiments, as shown in Tables V, VI and VII, indicate in a general way that the action of spleen extract on red cells is very much less than that of testicle extract. Each of the experiments has been repeated four times, and the results have been consistent. Text-fig. 1 shows the average degree to which the limit of fragility is brought down by the action of testicle and spleen extracts, as indicated by the maximum salt concentration at which hemolysis is produced.



TEXT-FIG. 1. Average of the results of the experiments showing the effect of testicle and spleen extracts on the fragility of red blood cells of rat, rabbit and guinea pig. Shaded areas represent complete hemolysis; cross-hatch, partial hemolysis; blank spaces, absence of hemolysis.

In addition to the foregoing, another experiment was carried out to test the action of rat testicle extract against the red blood cells of three *normal human individuals. The extract sharply lowered the corpuscular resistance in all three cases.*

In yet a third experiment with rabbit and rat red cells the effect of a purified bull testicle extract, which had proved very active in causing a spread of India ink through the subcutaneous tissue, was tested. The same lowering of corpuscular resistance was noted.

Finally we endeavored to detect a possible effect of rat testicle extract on guinea pig blood *in vitro*, by injecting the extract intravenously and comparing the normal resistance of the red cells with their resistance 2, 3 and 5 hours after the injection. No difference could be demonstrated. Negative results were also obtained when sheep blood cells, after a contact of 1 hour with rat testicle extract, were added to fresh guinea pig serum, or, conversely, when they were added to rat testicle extract after having been sensitized by the specific amboceptor.

DISCUSSION

It can be concluded from these experiments that testicle extract definitely increases red cell fragility, this action being more or less intense according to the animal species to which either testicle or red cells belong. Whatever the mechanism of any process of hemolysis, in the final analysis it is always through a modification of the permeability of the red cells that hemoglobin escapes into the surrounding liquid. Testicle extract has plainly the power *in vitro* to increase this permeability. The observation gains in significance when considered in the light of previous reports (1, 2, 5, 6), showing that testicle extract increases tissue permeability. That the factor which enhances the activity of infectious agents, the so called Reynals factor, is responsible for these effects has yet to be proved. But all the evidence thus far collected is in line with the view that it is responsible, and that the Reynals factor enhances the effects of viruses and bacteria through the local changes in cell permeability that it brings about in the tissues of the host.

The fact that spleen extracts have little or no power to lower the level of red cell resistance is consonant with the observation of Duran-Reynals (1) that these extracts fail to enhance the infectivity of viruses and bacteria, to increase the spread of inert particles through the tissues.

Many authors have been interested in whether the spleen is endowed with hemolytic power. The problem is important from the point of view of the pathogenesis of certain forms of anemia and jaundice, and also in relation to the much debated question of the origin of blood complement. A hemolytic power of the spleen has yet to be conclusively demonstrated. Korschum and Morgenroth (8), Donath and Landsteiner (9), Metschnikoff (10), Achard, Foix and Salin (11) and others have found in splenic extracts but a very slight hemolytic power, no greater than that shown by various other organs. However, the technique generally used in these experiments was simply to mix spleen extracts and red cells and to observe whether or not hemolysis occurred. In our experiments spleen extracts were found to have only a very slight power of lowering corpuscular resistance.

Others have noted—and we can confirm the observation—that occasionally spleens are found which exhibit a marked hemolytic power. Whatever the reason for this power, it does not compare with that consistently shown by testicle extracts.

CONCLUSIONS

Testicle extract possesses a notable power to increase red cell fragility. Testicle extracts of the rat, rabbit and guinea pig all exhibit this property, which is most pronounced with the extract from the rat and least so with that from the guinea pig.

Splenic extract does not possess the property, or possesses it only to a very slight degree.

These results support the hypothesis that the factor in testicle extract which enhances infections (the Reynals factor) does so by altering the permeability of the host tissue.

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CRITERIA OF THE AGE OF LYMPHOCYTES IN THE PERIPHERAL BLOOD

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PLATES 25 TO 27

(Received for publication, May 5, 1931)

Unlike most of the other cells in the adult body, all those found normally within the circulating blood continue throughout life to develop from primitive forms. In any analysis of the blood in disease, it is of the greatest importance to be able to determine the state of maturity of the cellular elements. The present work is concerned with criteria of the age of the lymphocyte.

There are two principal sites of origin for the blood cells: the bone marrow and the lymph nodes. Most of our present knowledge concerning the maturation cycle and the relative age or youth of cells in the circulation has been derived from studies of the erythrocytes and granulocytes arising in the bone marrow. By noting variations in certain cytoplasmic (1, 2) or nuclear (3–6) criteria, the relative age of these cells can be estimated and valuable information be obtained. The studies thus far recorded of lymphocytes and lymphatic tissue have not been of a character to contribute materially to the establishment of acceptable criteria of maturation for this strain of white blood cells. A search of the literature reveals some attempts to link the so called lymphocyte with the other blood cells as a hemoblast or precursor in the development of the various well recognized definitive types. Arneth (7), following his work on the neutrophilic granulocyte, published an index of nuclear variation in the lymphocytes to which he attached maturative significance. His studies have not received the verification and acceptance accorded to his observations on the polymorphonuclear leucocyte.

There are several reasons why the search for recognizable criteria of maturation in the lymphocyte has not been pressed. One must assume to begin with that among the mononuclear cells called "lymphocytes," some, if not all, arise as immature lymphocytes (lymphoblasts), and mature in a definite orderly way, to degenerate eventually without dedifferentiation. This assumption involves indorsement, at least in part, of the polyphyletic doctrine of cell evolution in relation to the lymphocyte, as it is obviously necessary for a cell to have a definite life cycle without change to other cell types before any conception of an orderly maturation can be entertained. The justification for such an assumption in relation to the lymphocyte will be evident as the experimental data which are to follow are introduced.

Another difficulty of the problem lies in the physical character of the lymphocyte itself, in that there is no apparent elaboration of specific structures in the cytoplasm. The development of granules in the cytoplasm and of nuclear polymorphism in the granulocyte, of a rosette of vacuoles stainable with neutral red in the monocyte, of phagocytic vacuoles without pattern in the clasmatocyte, and of hemoglobin in the red blood cell have all served as objective criteria upon which to base identification and a study of maturative phases. The lack of comparable criteria has been an obstacle to the recognition of age states in the lymphocyte, accustomed as we are to look for specific products in specific cells. It is evident that criteria of a different character must be sought for.

A third difficulty is to be found probably in the physiology of the lymphocytogenic tissues. Because of their anatomical distribution, it is highly probable that many of the lymphocytes perform their function within the tissue in which they arise. We may expect functionally mature lymphocytes to be intermingled with cells of varying degrees of immaturity in lymph nodes. It is impossible on the basis of present knowledge to examine lymphatic tissue, as in the case of bone marrow, with the assurance that there will be a preponderance of immature types with which to compare those forms in the circulating blood.

Finally, the uncertainty which exists concerning the exact structure of lymphatic tissue contributes to the complexity of the problem. Although most authors believe that the germinal center of Flemming is the lymphocytogenic focus, others interpret such so called germinal centers as reaction areas which do not participate in the origin of the lymphocyte.

Possible Criteria of Age

The following characteristics of blood cells may conceivably be of use in estimating the age of lymphocytes.

1. Basophilia.—In 1898 Pappenheim (8) noted that the cytoplasm of young myelocytes is very basophilic, and since then the association of basophilia with youth of the granulocytes has been noted by many investigators. The same fact has been observed for red blood cells. The "reticulation" in young red blood cells is merely a precipitation of basophilic substance (1, 2, 9–11); and in the reticulated cell counts we have an example of the use of the presence of basophilia as an index of immaturity. A careful application of the criterion to lymphocytes has apparently not been made.

2. *Mitochondria.*—That mitochondria are present in large numbers during the maturation of young blood cells other than lymphocytes is generally accepted (12–14); and in an excellent review, Cowdry (15) states that senescence is associated with diminishing numbers of mitochondria in the cytoplasm of cells in general. It should be mentioned, however, that increased numbers of mitochondria are associated with the activity of secretory cells.

3. *Size of the Cell.*—Hematologists have most frequently used cell size as a criterion of the age of lymphocytes. But how uncertain this has proved is shown by the fact that some have regarded the large (16, 17) and some the small (7, 18–20) lymphocyte as the younger cell, while others hold that size is no criterion of age at all (21). It is significant that most of the investigators studying fixed, stained specimens adhere to the belief that the large cells are the older types, whereas those who follow the supravital technique hold the reverse.

4. *Miscellaneous Features Characterizing the Lymphocyte.*—Here may be listed motility, vacuoles, chromatin content of nucleus, proportion of nucleus to cytoplasm, shape of nucleus (indentations), and azur granulation. The possible significance of these characteristics will be brought out further on.

Evaluation of Criteria

On analogy with the other cells of the blood, an estimation of the mitochondria in the supravital technique and of the degree of basophilia in the fixed films would seem most likely to afford criteria of value in the estimation of the age of the lymphocyte.

To test out this possibility, it is necessary to analyze the lymphocytes with respect to these criteria (1) in bloods which may be expected to show a normal distribution of age types, and (2) in bloods obtained when a physiological or pathological hyperplasia of lymphoid tissue should lead to increased numbers of immature types in the circulation. Of the criteria, basophilia is the most easily and accurately determined.

1. *Basophilia.*—With the supravital technique (17, 22) basophilia is detected by a grayish yellow hue or cast to the cytoplasm. In very basophilic cells this color approaches the hemoglobin tint of mature red corpuscles. A difficulty exists, however, in estimating relative basophilia in all the lymphocytes in supravital preparations, because of the very small amount of cytoplasm in the small

cells. *Only rarely can this criterion be applied except in intermediate and large lymphocytes, and since the small cell usually predominates, the factor of basophilia cannot be analyzed uniformly in the supravital preparations.*

Fixed and stained films furnish the best means for studying basophilia. Nearly all of the Romanowsky dye combinations bring out the basophilia clearly; but a combination of Wright and Giemsa stains (23) most clearly indicates its finer degrees. The usual Wright's staining method is followed, without washing, by a floating of the cover slip, stained side down, in diluted Giemsa (15 drops Giemsa plus 10 cc. distilled water at pH 6.5–7.0) for about 4 minutes. The cover slips are then blotted and mounted. The cytoplasm of lymphocytes usually does not take the stain uniformly; there may be condensations and rarefactions or an appearance of granulation. This is probably due to a precipitation of the basophilic substance comparable to the "reticulation" of red cells. Care must be observed to select fields for counting in which the spread is very thin, with no cells overlapping. It is also of prime importance that the stain be working properly, as it is useless to attempt to grade cells on basophilia in a poorly stained specimen.

After accustoming the eye to the differing shades of blue which this stain imparts to the cytoplasm, one can divide the lymphocytes arbitrarily into three types: The very basophilic, the moderately basophilic, and the slightly or not at all basophilic. The first type (Figs. 1 and 2) reacts with a color such as that obtained when a mark is made on white paper with a blue crayon (hereafter designated as "Y" types); the second type (Figs. 3 and 4) is "sky blue" (designated "M" types); while the third (Figs. 5, 6, and 7) is tinged scarcely at all with blue or may even react with a faint pinkish tinge to the cytoplasm ("O" types). Because of the gradual transition between the shades of color, it may be difficult at times to classify a cell, but with a little experience one learns to divide them rather sharply into the three classes. The smallest fragment of cytoplasm in a small lymphocyte is sufficient to make the classification on the basis of basophilia.

As the first step toward evaluating basophilia as a criterion of age, an analysis of the lymphocytes in a series of normal adult human and rabbit bloods was made. A total of 100 to 200 cells of all types were counted in the fixed smears and a percentage computation was made for each of the three degrees of basophilia. Tables I and II give the data procured.

In Table I the results obtained in blood specimen R 612C are omitted from the averages, as it was found that this man, an X-ray worker, was subject to recurring periods of lymphopenia, a fact discovered as a direct result of the finding in this survey.

It will be seen that normal bloods, both human and rabbit, have relatively definite and stable percentage composition of basophilic lymphocyte types. The average formula (Y-M-O) which expresses decreasing degrees of basophilia is for 13 human blood samples, 5-49-46. For 14 normal rabbits this formula is 4.7-48-47.3. As the tables show, the individual variation from these formulae was remarkably little. The number of specimens studied is insufficient to enable one to accept the formulae as representative of normal physiological standards, but they are adequate for the purposes of the present investigation.

TABLE I

Basophilia and Comparative Size of Lymphocytes in Normal Human Blood

Specimen	Sex	Date	No. of lymphocytes per c.mm.	Percentage of basophilic types Fixed technique			Percentage of size types Supravital technique		
				Y forms	M forms	O forms	Small	Int.	Large
R 1497*	F	May 21	1,768	9	48	43	96	4	0
R 1498	M	May 21	1,749	6	64	30	56	21	3
R 1499	M	May 21	869	5	55	40	88	12	0
R 1500	F	May 21	1,539	4	54	42	80	12	8
R 1501	M	May 21	2,240	6	47	47	82	15	3
R 1502	M	May 21	3,081	3	56	41	59	41	0
R 1503	M	May 21	4,238	4	65	31	98	2	0
R 169F	M	Apr. 25	3,360	7	34	59	72	28	0
R 612C	M	Apr. 25	896	17	58	15	100	0	0
R 612W	M	Apr. 25	1,716	3	36	61	76	24	0
R 612P	M	Apr. 25	1,470	6	69	25	70	30	0
R 612L	M	May 20	1,608	4	32	64	84	16	0
R 1493T	M	May 20	854	8	35	57	86	14	0
R 1493M	F	May 20	1,001	3	50	47	92	8	0
Average (R 612C omitted).....			1,960	5	49	46	82	17	1

* These are serial numbers of the work of the department covering a term of years.

The second step was to examine bloods which might be expected to show an increase in immature lymphocytes. Since in new-born animals immature myeloid and erythroid cells are regularly found in the circulating blood, it is logical to expect an unusually great proportion of immature lymphocytes. Examination was made of 12 samples of blood from 6 rabbits the ages of which varied from 12 hours to 19 days, and the lymphocytes were graded according to basophilia as before. Owing to the extreme leucopenia in the young rabbits, only 25 to 50 cells of all types were counted. Table III contains the data thus secured.

Considering for the moment only the column containing the basophilic types, it is noted that the average formula (Y-M-O) is 38.8-53.3-7.9; that is, there is a marked shifting to the heavily basophilic types as compared with specimens of normal adult blood.

TABLE II

Basophilia and Comparative Size of Lymphocytes in Blood of Normal Rabbits

Rabbit	Date	No. of lymphocytes per c.mm.	Percentage of basophilic types Fixed technique			Percentage of size types Supravital technique		
			Y	M	O	Small	Int.	Large
R 1129*	3/12–4/14	3,045	6.7	47	46.3	92.6	6	1.4
R 1130**	3/12–4/21	6,079	3.7	50.6	45.7	90.7	7.8	1.5
R 1134**	3/12–4/14	2,623	11.1	54.4	34.5	87.8	8.6	3.6
R 1131	5/20	2,968	3	53	44	72	28	0
R 1132	5/20	13,778	2	56	42	99	1	0
R 1133	5/20	2,275	7	45	48	94	3	3
R 1135	5/20	4,033	15	56	29	88	9	3
R 1136	5/20	3,549	2	49	49	89	11	0
R 1137	5/20	2,460	2	50	48	83	14	3
R 1138	5/20	1,396	7	53	40	90	10	0
R 1139	5/20	7,864	3	44	53	98	2	0
R 1167	5/20	7,260	0	40	60	92	3	5
R 1169	5/20	4,902	0	26	74	94	6	0
R 1170	5/20	2,004	3	45	52	96	4	0
Averages............		4,590	4.7	48	47.3	90.5	8.1	1.4

* R 1129 represents average of 9 counts.

** R 1130 and R 1134 represent averages of 11 counts each.

Blood specimens from 20 rabbits with tuberculosis were next examined for possible changes in the cellular formula (Table IV). These tuberculous rabbits have been divided into two groups on the basis of the monocyte-lymphocyte ratio in the peripheral blood; eleven in which the M/L index was less than 0.50, probably indicating a minimum of tuberculous activity (24, 25), and nine in which this index was 0.50 or more, probably indicating progressive lesions. In the latter group it will be seen that there is a notable shift in the lymphocyte formula to the more basophilic types, 22 per cent, together with a relative lymphopenia, when compared with the group in which the M/L index was less than 0.50. In those cases in which the M/L ratio is highest (see R 1366, R 1309, and R 1103, in particular), the increase in number of basophilic lymphocytes is especially marked.

TABLE III

Percentage of Basophilic, Mitochondrial, and Size Types in the Blood of New-Born Rabbits

Rabbit	Age	Date	No. of lymphocytes per c.mm.	Percentage of basophilic types Fixed technique			Percentage of mitochondrial types Supravital technique			Percentage of size types Supravital technique		
				Y	M	O	Y	M	O	Small	Int.	Large
R 1377	4 days	3/8	864	15	78	7	38	38	24	70	30	0
R 1377	10 "	3/15	1,100	50	46	4	0*	100	0	88	12	0
R 1377	19 "	3/24	2,400	43	53	4	42	53	5	84	14	4
R 1378	4 "	3/8	810	47	47	6	0*	70	30	90	10	0
R 1378	10 "	3/15	840	59	36	5	24	62	14	95	5	0
R 1378	19 "	3/24	605	32	63	5	29	61	10	90	5	5
R 1379	4 "	3/8	594	30	65	5	0	57	43	34	66	0
R 1379	10 "	3/15	592	35	53	12	25	69	6	94	6	0
R 1379	19 "	3/24	1,620	46	32	22	15	55	30	96	4	0
R 1418	12 hrs.	3/31	192	25	50	25	25	50	25	100	0	0
R 1419	12 "	3/31	320	44	56	0	42	58	0	88	12	0
R 1420	12 "	3/31	260	40	60	0	0*	100	0	100	0	0
Averages.............			850	38.8	53.3	7.9	20.0	64.5	15.5	85.7	13.6	0.7

* Note that the difficulty in accurately estimating large numbers of mitochondria sometimes results in many or all the "Y" types being classified with the "M" types. This remark also applies to Table VI.

In general, this relationship between basophilia and the M/L index persists throughout the table.

Since it is generally believed that the lymphocyte is an important element in resistance to tuberculosis, it is logical to assume that in cases progressing unfavorably, cases that is to say with a high M/L index, the lymphopenia reflects a demand for lymphocytes which the lymphatic tissues cannot adequately meet, and under such circumstances the appearance in the blood stream of less mature types may be expected and is easily understood. In formulating this hypothesis, I am

TABLE IV

Percentage of Basophilic and Size Types of Lymphocytes in the Blood of Tuberculous Rabbits

Rabbit	M/L index	Date	No. of lymphocytes per c.mm.	Percentage of basophilic types Fixed technique			Percentage of size types Supravital technique		
				Y	M	O	Small	Int.	Large
R 1366	1.25	4/29	1,680	31	62	7	88	12	0
R 1309	1.21	4/21	2,590	53	41	6	93	7	0
R 1103	1.00	4/21	2,268	24	48	28	90	5	5
R 1152	0.93	5/19	2,380	13	60	27	84	16	0
R 1248	0.80	5/28	3,680	15	60	25	85	12	3
R 1262	0.60	4/28	4,865	9	52	39	94	3	3
R 1260	0.56	5/28	3,888	10	52	38	80	17	3
R 1381	0.56	4/28	1,927	18	53	29	90	5	5
R 1367	0.50	4/29	3,710	25	65	10	97	3	0
R 1383	0.43	4/28	3,220	11	57	32	83	17	0
R 1259	0.35	5/28	2,793	14	50	36	70	27	3
R 1112	0.33	4/30	3,663	13	38	49	76	24	0
R 1103	0.31	5/28	1,856	10	50	40	87	13	0
R 1184	0.22	5/28	2,808	13	44	43	91	7	2
R 1255	0.18	5/28	8,673	1	36	63	100	0	0
R 1389	0.18	4/28	3,753	11	63	26	93	7	0
R 1179	0.16	5/28	4,434	15	50	35	90	10	0
R 1269	0.15	5/28	3,054	5	52	43	93	7	0
R 1382	0.12*	4/1	5,964	26	60	14	60	30	10
R 1176	0.10	5/28	7,735	4	47	49	88	12	0
Average of all rabbits	0.497		3,747	16.0	52.0	31.9	86.6	11.7	1.7
Average of group with M/L index of 1.25–0.50 (9 animals)..........			2,998	22.0	54.7	23.2	89.0	8.8	2.1
Average of group with M/L index of 0.43–0.10 (11 animals)..			4,359	11.1	49.7	39.0	84.6	14.0	1.3

* Rabbit R 1382 died 2 weeks after this blood count was taken, indicating that this animal probably had advanced tuberculosis.

merely applying to the lymphatic tissues the same reasoning which has been applied to the myeloid tissues and found true in the case of pyogenic infections.

Further evidence to show that increasing basophilia is a criterion of increasing immaturity was obtained by the examination of blood from animals in which lymphoid hyperplasia has been artificially induced. Figs. 10–15 show the degree of hyperplasia induced in the lymphatic tissues of rabbits receiving parenteral injections of foreign protein (26). They show a multiplication of lymph follicles both in lymph nodes and in the spleen, together with an increase in basophilic forms and in mitotic figures in these tissues. The blood counts of these same animals are given in Table V.

The left half of Table V shows the extent of increase in the numbers of lymphocytes per cubic millimeter during the period of the injections. The basophilic formula at various times during the period of the injections is shown in the right hand section of the table. Expressed as an average of 25 examinations of the blood from 5 animals, this formula is 27.1-46.9-26, which again shows a marked shift to the more basophilic types. If the hypothesis is correct that the peripheral blood reflects the state of activity of the tissue from which its cells are derived, then it must be concluded that basophilia is a measure of immaturity of the lymphocyte.

2. *Mitochondria.*—If it be accepted on the basis of the above evidence that basophilia is directly related to youth in the lymphocyte, then it becomes possible to see if any other property, such as a high mitochondrial content, is linked with youth.

Blood specimens in normal adult, new-born, and tuberculous rabbits were examined for the purpose of obtaining direct comparison between these two factors. The intensity of basophilia was determined in Wright-Giemsa-stained specimens and mitochondrial content in supravital preparations. To preclude obtaining specimens of blood for comparison that might be dissimilar with respect to the lymphocytes, both preparations were made from portions of the same drop of blood by drawing it into the stem of a white cell diluting pipette and expelling small portions very rapidly, first for the supravital and then for the fixed preparations.

Mitochondrial content was determined by count whenever possible, at other times by rough estimation; and the classification was made as follows: Class Y represents cells with many mitochondria, that is, more than 20; Class M includes cells with from about 5 to 20 mitochondria,

TABLE V

Percentages of Basophilic Types of Lymphocytes in Rabbits with a Hyperplasia of the Lymphatic Tissues Induced by Protein Injections

Rabbit	Average Nos. of lymphocytes per c.mm. of blood during control and injection periods with date of autopsy		Date of count	Per cent basophilic types during injection period Fixed technique		
				Y	M	O
R 1157	Control period	2,477	2/21/30	27	51	22
	Injection period	3,911	2/26/30	59	30	11
	Date of autopsy	3/27/30	3/14/30	25	50	25
			3/19/30	27	59	14
			2/24/30	23	42	35
			3/26/30	24	39	37
R 1158	Control period	3,507	2/10/30	32	45	23
	Injection period	5,151	2/11/30	30	52	18
	Date of autopsy	2/21/30	2/19/30	33	38	29
			2/21/30	38	43	19
R 1160	Control period	3,179	2/26/30	37	43	20
	Injection period	5,599	3/ 7/30	20	51	29
	Date of autopsy	3/20/30	3/10/30	10	41	49
			3/14/30	23	40	37
			3/19/30	32	43	25
R 1163	Control period	3,208	2/14/30	20	57	23
	Injection period	6,500	3/ 7/30	16	60	24
	Date of autopsy	4/ 4/30	3/10/30	20	47	33
			3/19/30	18	54	28
			3/24/30	15	52	33
			3/26/30	31	47	22
			4/ 2/30	18	54	28
			4/ 4/30	13	46	41
R 1185	Control period	2,504				
	Injection period	5,983	4/25/30	62	28	10
	Date of autopsy	6/20/30	4/28/30	25	59	16
Averages....	Control period	2,975		27.1	46.9	26
	Injection period	5,429				

and Class O cells with from 0 to 5. The corresponding groups of lymphocytes arranged according to basophilia of the cytoplasm as determined in the fixed films were classified as before; that is, Class Y, deeply basophilic, Class M, moderately basophilic, and Class O, slightly or not at all basophilic. Tables III and VI present the data so obtained.

It is impossible to obtain accurate estimations of mitochondrial content, partly because of the physical difficulties involved in counting these bodies and partly because of the fact that some of the mitochondria occasionally fail to take the dye, while some fading of the stain often occurs. Tables III and VI are therefore presented to show trends, not exact quantitative relationships. As such, the averages in these tables demonstrate clearly that when the trend of the lymphocytes is towards increased cytoplasmic basophilia there is also a corresponding shift towards increased mitochondrial content. This same fact is demonstrated in Table VIII, Columns 6 and 7, in which basophilia and mitochondrial content of each of 75 large lymphocytes are directly compared by supravital examination.

3. Size of the Cell.—With the demonstration that increasing mitochondrial and basophilic content of the cytoplasm of lymphocytes are found together and that they are criteria of increasing immaturity, it becomes important to test the relation that cell size bears to these indices. The facts to be determined are: (1) The relation, if any, between increasing immaturity and the increase or decrease in numbers of the large cells, and (2) the determination of the status of the large lymphocyte with respect to its position in the maturative cycle. As a prerequisite for this study it was necessary first, to consider the measurements obtained as such, and second, to determine if the procedure of pulling and fixing cover slips distorts the size and appearance of the cell.

Among many hundreds of lymphocytes measured, the smallest encountered was 5.2 micra in diameter, and the largest 20.0 micra. The usual allocation of size to type has been given as 6μ–9μ for the "small" lymphocyte, 9μ–12μ for the "intermediate," and $12\mu+$ for the "large" lymphocyte (16, 20, 21, 27). It was found, however, that a great number of cells, when studied by the supravital method, measured slightly over 9μ, with very few between this size and 10.5μ.

For this reason the following standards of size were adopted in the present study: up to 10μ the small lymphocyte, 10μ–12μ the intermediate, and 12μ+ the large lymphocyte.

TABLE VI

Comparison by Percentage of Mitochondrial and Basophilic Types of Lymphocytes in Three Normal and Five Tuberculous Rabbits

Rabbit	Date	Pathology	Percentage of basophilic types Fixed technique			Percentage of mitochondrial types Supravital technique		
			Y	M	O	Y	M	O
R 1129	3/12	None	7	50	43	3	55	42
R 1129	3/14	"	4	46	50	3	51	46
R 1129	3/17	"	10	44	46	6	73	21
R 1129	3/19	"	5	47	48	5	47	48
R 1129	3/24	"	7	57	36	7	57	36
R 1129	3/28	"	6	45	49	8	52	40
R 1129	3/31	"	10	37	53	4	61	35
R 1129	4/2	"	5	52	43	5	52	43
R 1130	3/12	"	3	60	37	20	60	20
R 1130	3/14	"	8	61	31	0	50	50
R 1130	3/17	"	2	48	50	6	60	34
R 1130	3/19	"	2	55	43	3	58	39
R 1130	3/24	"	5	56	39	3	65	32
R 1130	4/2	"	5	49	46	8	55	37
R 1134	3/12	"	11	46	43	18	43	39
R 1134	3/14	"	12	62	26	0	74	26
R 1134	3/17	"	20	47	33	9	69	22
R 1134	3/19	"	17	60	23	17	61	22
R 1134	3/24	"	16	43	41	8	60	32
R 1134	4/2	"	12	50	38	6	63	31
R 1185	4/25	Tbc.	62	28	10	24	62	14
R 1176	4/2	"	30	60	10	0	80	20
R 1366	4/29	"	31	62	7	18	75	7
R 1367	4/29	"	25	65	10	30	61	9
R 1382	4/1	"	26	60	14	11	60	29
Averages for R 1129...........			6.7	47.2	46.1	5.1	56.0	38.9
Averages for R 1130...........			4.1	54.8	41.1	6.6	58.0	35.4
Averages for R 1134...........			14.6	51.3	34.1	9.6	61.6	28.8
Averages for tbc. rabbits.......			34.8	55.0	10.2	16.6	67.6	15.8

Within limits, there is little or no relation between the size of the lymphocyte as obtained in the fixed smear and the real size of that cell in the circulating blood. Evidence in support of this statement is offered in Table VII. This represents a comparison of the measured gross diameters of the lymphocytes encountered in five separate differential counts of five different normal human bloods. Portions from the same drop of blood were used in making both supravital and fixed spreads.

TABLE VII

Number of Lymphocytes of Various Sizes Found in Counting 100 White Blood Cells in Supravital and Fixed Preparations of Blood from Five Normal Human Adults

Blood specimen	Technique	Gross cell diameters in micra															
		5–6	6–7	7–8	8–9	9–10	10–11	11–12	12–13	13–14	14–15	15–16	16–17	17–18	18–19	19–20	20+
R 1497 Female–age 58	Supravital	—	6	13	3	4	1	—	—	—	—	—	—	—	—	—	—
	Fixed	—	—	—	1	8	1	4	3	2	6	2	—	—	—	—	—
R 1498 Male–age 28	Supravital	—	—	6	8	11	6	1	1	—	—	—	—	—	—	—	—
	Fixed	—	—	—	4	5	4	10	7	0	2	1	—	—	—	—	—
R 1499 Male–age 31	Supravital	1	2	2	3	6	0	2	—	—	—	—	—	—	—	—	—
	Fixed	—	—	—	—	1	2	4	6	1	2	1	—	—	—	—	—
R 1500 Female–age 27	Supravital	—	3	9	1	8	1	3	1	0	1	—	—	—	—	—	—
	Fixed	—	—	—	1	3	4	10	6	0	2	1	—	—	—	—	—
R 1501 Male–age 33	Supravital	2	4	8	6	6	0	4	0	0	1	—	—	—	—	—	—
	Fixed	—	—	—	1	5	2	6	6	2	6	2	0	0	0	2	—
Totals	Supravital	3	15	38	21	35	8	10	2	0	2	—	—	—	—	—	—
	Fixed	—	—	—	7	22	13	34	26	5	18	7	0	0	0	2	—
Per cent	Supravital	83					13		4								
	Fixed	20					35		45								

It is obvious that there is a great preponderance of large forms in the fixed films that does not occur in supravital preparations. Thus, only 4 per cent of the lymphocytes in the living state measured over 12 micra, whereas in the fixed films the corresponding percentage was 45.

This means, that in viewing a large lymphocyte in the fixed preparation, one cannot tell the size of that cell when in the circulating blood. The explanation is suggested that some cells, being more fragile than others, are flattened out by the technique usual in pulling cover slips with consequent distortion of size. Others, being more elastic and coherent, resist more effectually this mechanical factor. In cases of decrease in cell size, it can be supposed that contact with fixative has resulted in shrinkage of the cells.

Having determined that the size of cells in fixed specimens is not comparable with that of living ones, it became necessary to compare the percentages of the three cell sizes, as determined by supravital examination, with the percentages of the basophilic types obtained in fixed films, if one was to establish any relation between size and state of maturity. Such comparisons in bloods from normal human adults (Table I), normal adult rabbits (Table II), new-born rabbits (Table III), and tuberculous rabbits (Table IV) show that an increase in immature forms may occur without any notable shift in size. However, an analysis of these tables indicates that in some instances an increase in young forms was associated also with an increase in the larger types of cells (compare average of size types in Tables II, III, and IV and individual counts of R 1377 and R 1378 on March 24 of Table III, and R 1382 on Table IV).

A study of large lymphocytes was now undertaken to determine if they are predominately young or old. To determine this point, 75 large lymphocytes were measured in supravital specimens and observations were also made on their degree of basophilia and mitochondrial content and on certain other features of the living cell. The results obtained are shown in Table VIII, Columns 2, 6, and 7.

Analysis of Table VIII indicates that most but not all of the large lymphocytes have a marked basophilia and contain large numbers of mitochondria. Those that are deeply basophilic and with nearly maximum numbers of mitochondria (+++ and ++++) make up 70 per cent of the large lymphocytes examined, whereas the cells deficient in these respects (0 and 1+) constitute only 15 per cent of those surveyed. As an example of the latter group, a very large cell (No. 27), measuring 16.1μ, had a cytoplasm entirely deficient in signs of basophilia and mitochondria. Since these two characteristics,

TABLE VIII

Supravital Characteristics of Large Lymphocytes in the Blood of Rabbits

Cell No.	Gross diameter	Approximate ratio of diameter of nucleus to cell	Chromatin content of nuclei	Approximate depth of indentation of nucleus μ	Basophilia of cytoplasm	Mitochondria	Neutral red vacuoles	Nucleoli	Non-staining refractive vacuoles
1	14.9	0.90	++	F	++++	++++	+	2	0
2	13.3	0.60	++	F	++++	++++	++	0	0
3	15.0	0.70	++++	1.6	+++	+++	+	0	0
4	15.0	0.55	++++	Round	++++	++++	++	0	0
5	14.5	0.60	+++	2.6	++++	+++	+	0	0
6	16.6	0.75	++	1.8	++++	++++	++	0	0
7	15.0	0.70	++	1.2	+++	++	+	0	0
8	17.5	0.60	++++	0.5	++++	++++	++	0	0
9	14.5	0.70	++	0.5	++++	+++	++	0	0
10	15.0	0.80	++++	1.0	+	++	+++	0	0
11	13.2	0.55	++	1.6	+++	++	++	0	0
12	15.7	0.60	++	0	++++	+++	+	0	0
13	12.8	0.60	+	1.6	++++	++	+++	0	0
14	15.0	0.88	++++	Round	+++	+++	++	0	0
15	16.0	0.70	+	Round	++++	+++	+++	0	0
16	15.0	0.70	++	F	++++	++++	+	1	0
17	14.5	0.65	++++	2.2	++++	++++	++	0	0
18	13.0	0.75	+	1.5	+++	+++	++	0	0
19	13.0	0.75	++++	0	+++	+++	++	0	0
20	13.0	0.75	++++	F	+++	+++	++	0	0
21	16.0	0.60	++++	Oval	++++	++++	++	0	0
22	13.0	0.60	+	F	++	++	+	0	0
23	15.0	0.75	++	5.0	+++	++	+	0	0
24	14.0	0.70	++	4.0	++++	+++	++	0	0
25	19.5	0.90	++	4.5	++++	++++	+++	0	0
26	14.4	0.75	+	Round	++	++	+++	0	0
27	16.1	0.30	+++	Round	0	0	0	0	1
28	15.0	0.70	++	F	++	++	+	0	0
29	14.5	0.75	++	5.0	+++	+++	++	0	0
30	16.0	0.70	++	0.5	++++	++++	+++	0	0
31	14.5	0.70	++	2.0	+++	+++	+	0	0
32	14.4	0.45	+	Round	0	0	++	0	++++
33	15.0	0.50	+	F	+	+	++++	0	+
34	20.0	0.75	++	Oval	++++	++++	+++	0	0
35	16.5	0.95	++	Round	++++	++++	+++	1	0
36	13.0	0.85	++	Round	++++	+++	0	0	0
37	16.0	0.90	++	F	+++	+++	+++	0	0
38	14.5	0.50	+	Round	0	0	0	0	++++

F = flattened.

TABLE VIII—*Concluded*

Cell No.	Gross diameter	Approximate ratio of diameter of nucleus to cell	Chromatin content of nuclei	Approximate depth of indentation of nucleus	Basophilia of cytoplasm	Mitochrondria	Neutral red vacuoles	Nucleoli	Non-staining refractive vacuoles
				μ					
39	16.0	0.75	+	Oval	++	++	++++	0	0
40	16.1	0.55	+	2.5	++	++	++++	0	0
41	19.0	0.80	++++	F	++++	++++	+++	0	0
42	14.4	0.90	++	1.0	++++	++++	++	0	0
43	15.0	0.60	++	Oval	+++	+++	++	0	0
44	14.5	0.70	++++	1.0	+++	+++	++	0	0
45	13.5	0.85	+++	Oval	++++	++++	+	0	0
46	15.0	0.75	+	F	+++	++	++	0	0
47	13.0	—	—	—	++++	++++	0	0	0
48	13.5	0.70	++	5.0	++	++	++++	0	0
49	16.0	0.55	+++	4.0	++++	++++	0	0	0
50	16.0	0.60	+	4.0	++	++	+	0	0
51	13.0	0.60	++	Oval	++++	++++	+	0	0
52	13.0	0.70	++	F	++	++	++	0	0
53	13.0	0.60	++	F	+	+	++++	0	++++
54	13.0	0.70	++	5.0	++	++	++++	0	0
55	15.0	0.65	++	2.0	++	++	++++	0	0
56	13.0	0.70	++	Round	++++	++++	+++	0	0
57	12.8	0.70	+	F	0	0	++++	0	0
58	15.0	0.70	++	F	++++	++++	+++	0	0
59	12.8	0.80	++++	Round	++++	++++	0	1	0
60	14.5	0.55	+++	Round	0	0	++	0	+
61	12.8	0.70	++	Oval	++++	++++	0	0	0
62	17.5	0.65	+++	Round	+++	+++	++	0	0
63	12.0	0.70	++	Oval	++	++	++++	0	0
64	16.0	0.75	++	Oval	++++	++++	+	0	0
65	14.5	0.70	++	F	+++	+++	++++	0	0
66	14.5	0.75	++	F	+++	+++	++++	0	0
67	14.5	0.60	++	3.0	+++	+++	++++	0	0
68	14.5	—	—	—	++++	++++	+	0	0
69	13.0	0.70	+++	Oval	+	+	++++	0	++
70	11.2	0.60	+++	Round	0	0	+	0	++++
71	14.5	0.60	+++	F	++	++	++	0	0
72	13.0	0.65	+	Oval	+	0	0	0	++
73	14.4	0.70	++	Oval	+++	+++	++	0	0
74	14.5	0.90	++++	Round	++++	++++	++	1	0
75	16.0	0.70	++++	Round	+++	+++	++	0	0

Cell 69 changed to Cell 70 in 15 minutes under observation.

Cell 72 was watched for 1⅓ hours and no change in morphology occurred.

when present, have been shown to be associated with immaturity, it can be concluded that, although the majority of large lymphocytes of the supravital films are young, cells may be large at any age. The same holds true for the erythrocyte and leucocyte.

4. Miscellaneous Features Found in Lymphocytes.—Amongst the features detectable in the lymphocytes of supravital preparations, there remain to be evaluated the shape of nucleus (indentations), the relative size of nucleus to gross cell size, chromatin content of the nucleus, non-staining refractive vacuoles, and content of neutral red vacuoles. For the association of these features with mitochondrial content, basophilia, and cell size, reference is again made to Table VIII. The number of neutral red bodies, mitochondria, and refractive vacuoles were counted or estimated on a basis of 0 to 5 = +; 5 to 15 = ++; 15 to 30 = +++; over 30 = ++++. The amount of indentation of nucleus was measured or estimated; basophilia and amount of chromatin in the nucleus were estimated as +, ++, +++, and ++++.

Analysis of Table VIII with respect to these features indicates: (*a*) That the shape of the nucleus of the lymphocyte (presence of indentation and its degree) does not bear any relationship to the number of mitochondria or the degree of basophilia, and hence is unrelated to age in direct contradiction to Arneth's hypothesis (7), whereas the chromatin content of the nucleus does appear to be greatest in those cells deficient in the cytoplasmic criteria of youth. (*b*) That in general, in the large lymphocytes, the greater the amount of cell occupied by the nucleus, the more basophilic the cytoplasm. (*c*) That nucleoli are visible only in the nuclei of cells with deeply basophilic cytoplasm and sparse chromatin. It is probable that only large nucleoli are visible by this method of examination, as very small nucleoli are likely to be obscured from view by nuclear substance. (*d*) That non-staining, refractive vacuoles are found only in cells devoid of both mitochondria and basophilic substance; whereas the presence and number of neutral red vacuoles do not seem to bear any relationship to either of these features.

Azur granulation is seen in fixed preparations, but not in the supravital. The observations made in the present study have indicated that these granules are associated most often with the lesser basophilic types. However, one cannot be too certain that they are entirely limited to this older cell type, because the dark staining of the cytoplasm in the young basophilic cells may make it impossible to detect them when present. The fact that no cytoplasmic structure of the living lymphocyte can be identified with the azur granule of the dead cell suggests that the latter is a precipitation brought about by fixation, possibly evidencing some functional activity of the mature cell.

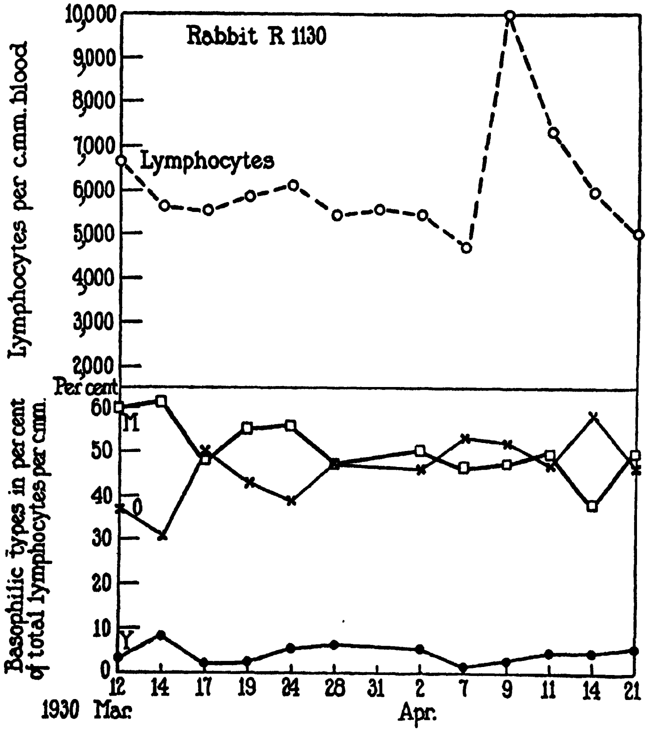

CHART 1. There are four curves on this chart to be contrasted. The uppermost shows the fluctuations in total number of lymphocytes; the curve labelled "O" designates the percentage of old cells (least basophilic); "M" refers to the mature (moderately basophilic) types; while the curve "Y" at the bottom of the chart indicates the percentage of young or heavily basophilic types.

Relation of Lymphocytosis to Cellular Immaturity

Arneth's dictum that the total number of granulocytes in the blood is not necessarily a criterion of the activity of the bone marrow has been amply confirmed. It is of interest to analyze the number of lymphocytes circulating in the peripheral blood as an index to activity of the lymphoid tissue. Since it is now evident that the greater the basophilia the greater the immaturity of lymphocytes, one is enabled to make the analysis mentioned by comparing directly the percentage of the basophilic types with the total numbers of circulating cells. For this purpose, observations were made three times per week for 1 month on the lymphocytes in the blood of three stock rabbits. Chart 1 represents the findings in one of these animals. It is typical of all three.

Chart 1 shows clearly that the total number of lymphocytes in the peripheral blood is not necessarily a criterion of a percentage increase therein of young cells. Note especially that the high peak in total lymphocytes on April 9th showed no corresponding fluctuation in the percentage of basophilic types.

DISCUSSION

The present study shows that in the maturing lymphocyte changes take place in number of mitochondria and degree of basophilia, which have the same general character as those occurring during the life of the other cells of the blood.

Although basophilia is manifestly a criterion of youth, the absence of it in a given cell does not necessarily mean that the cell is mature. No one criterion enables one to pronounce upon this point. In other cell strains some of the recognized characteristics of youth may be absent in elements of undoubted immaturity. For example, occasionally there are seen early myelocytes that are motile (about 60 per cent in one case of myelocytic leucemia recently observed); mature neutrophilic leucocytes with very few specific granules; myelocytes without appreciable numbers of mitochondria or with little basophilic substance. From this experience with cell types other than the lymphocyte, it would seem that in the case of the lymphocyte as in that of other cell types, all of the criteria of immaturity must be taken into account in an age classification. Basophilia is the most constant and reliable criterion.

It now seems certain that each strain of blood cells can be traced back to a simpler form characterized by a more basophilic cytoplasm.

The widespread distribution throughout the body of basophilic mononuclear cells gave rise to Pappenheim's concept of a "lymphoidocyte" or indifferent polyvalent primitive cell. The monophyletic theory of cell origin has been based upon this conception. Although the decreasing basophilia which characterizes the cytoplasm of the lymphocyte from youth to senility is wholly analogous to that seen in the other types of developing white blood cells, nevertheless the demonstration of an early basophilic form in this series, need not necessarily be interpreted as support for the monophyletic hypothesis.

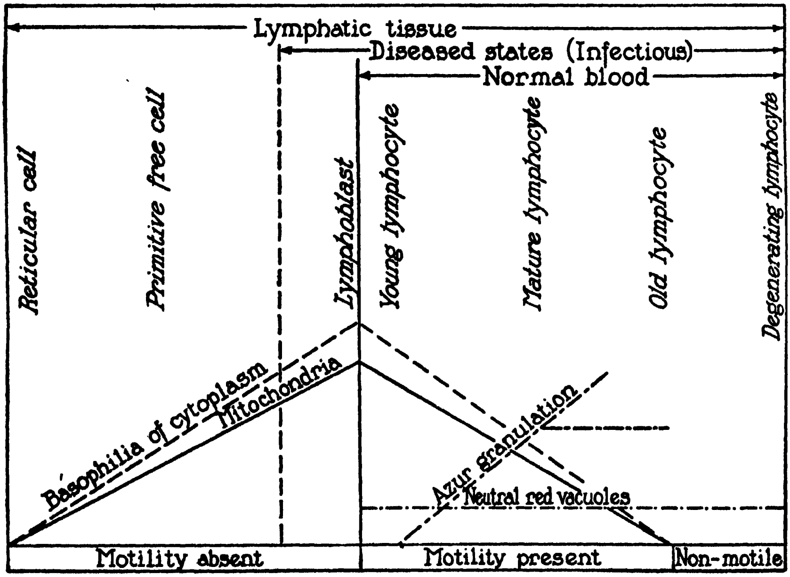

CHART 2. Diagram of the maturation of lymphocytes

The dualists have always held that each cell strain has its own "cytoblast," which matures only in the lineage peculiar to that specific cell type, while the monophyletic school have given the "lymphocyte" at all times multipotential powers of differentiation. The recognition of lessening basophilia of the lymphocyte until in the degenerating form the cytoplasm shows no basophilia whatever, establishes the lymphocyte as an independent definitive cell strain existing in parallel with the other blood cells, not preliminary to them. It will be recalled that such an assumption was necessary to the undertaking of the present work; and it has been justified. A graphic representation of the changes taking place during the life of the lymphocyte is now possible. Chart 2 is such a representation, patterned after the schema of Sabin (13) for the red cells and granulocytes.

The failure to recognize basophilia as an expression of youth of the lymphocyte has often resulted in a designation of young forms as plasma cells. All possible gradations in basophilia have been encountered within the lymphocytic series as is indicated by Chart 2.

Unna's original concept (28) of the plasma cell included numerous basophilic forms. Von Marschalkó (29), however, sharply limited this term to a specific group of cells showing a relatively small nucleus excentrically placed, containing chromatin particles usually arranged radially (*Radkern*), with a central clear area in the protoplasm opposite the nucleus, and with very basophilic cytoplasm. The irritation forms of Türk (18) are usually considered as plasma cells (Naegeli (21)), but obviously if one accepts the definition of von Marschalkó these cells cannot be plasma cells.

Arneth's expression "shift to the left" (4) had its inception in the observation that under pathological conditions there occurs an increase in the proportion of those granulocytes showing fewer lobes to the nucleus, *i. e.*, younger cells. In a larger sense, "shift to the left" has come to express a shift toward the younger stages in the maturation cycle of some other blood elements, and applied in this broader sense, it is highly useful. When there occurs an increase in the proportion of older forms, the term "shift to the right" is applicable. It is evident from the present work that the terms can be used with respect to the lymphocyte.

SUMMARY

The study of blood from rabbits with normal and with hyperactive lymphatic tissue reveals, in the latter, a greater percentage of lymphocytes with heavily basophilic cytoplasm and numerous mitochondria. This indicates that cytoplasmic basophilia and mitochondrial content can serve as criteria of the degree of maturity of the lymphocyte, these characters having the same significance in this relation as obtains with other blood cells. Basophilia is the more evident and reliable indicator of youth of the cells. The classification of lymphocytes into three groups, according to degree of basophilia, has yielded figures which show the proportions of the three to be relatively stable in blood from normal adult human beings and rabbits.

Size is not strictly a function of age in lymphocytes. Moreover, there is no correspondence in the size of lymphocytes in supravital films and in fixed specimens obtained by the "cover glass" method. There is a change of size during fixation. Although lymphocytes of intermediate and large size may be of any age, in supravital preparations the majority are young cells, whereas in fixed films the reverse obtains. The small lymphocyte may be of any age in specimens examined by either technique.

The total number of lymphocytes circulating at any given time is not necessarily an index to lymphoid activity.

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EXPLANATION OF PLATES

PLATE 25

FIG. 1. A large, very young lymphocyte from the peripheral blood of Rabbit R 1185, Apr. 25, 1930. Wright-Giemsa stain. Observe the granular character and extreme degree of basophilia of the cytoplasm. This is the "Y" type of cell mentioned in the text. Diameter of cell was 16.0μ.

FIG. 2. A small lymphocyte showing the same characters. From Rabbit R 1381, Apr. 28, 1930. Diameter of cell was 8.5μ.

FIG. 3. An intermediate lymphocyte from the peripheral blood of Rabbit R 1185, Apr. 25, 1930. Wright-Giemsa stain. Note the color of the cytoplasm. This is the "M" type of cell described in the text. Diameter of cell was 11.5μ.

FIG. 4. A large lymphocyte showing the same features. From peripheral blood of Rabbit R 1185, Apr. 25, 1930. Diameter of cell 16.0μ.

FIG. 5. A large lymphocyte from the peripheral blood of Rabbit R 1152, May 19, 1930. This is an old cell and is designated as the "O" type in the text. Note the lack of basophilia of the cytoplasm. One azur granule is to be seen near the top of the cell. Wright-Giemsa stain. Diameter of cell was 16.0μ.

FIG. 6. An intermediate lymphocyte showing the same features. From peripheral blood of Rabbit R 1152, May 19, 1930. Diameter of cell was 11.8μ.

FIG. 7. A small lymphocyte showing the same features. From the peripheral blood of Rabbit R 1176, May 28, 1930. Diameter of cell was 8.0μ.

FIG. 8. A red blood cell from the peripheral blood of a rabbit stained by the Wright-Giemsa technique,—for purposes of comparison.

PLATE 26

FIG. 9. Section of inguinal lymph gland of Rabbit R 1160. Removed at biopsy before protein injections. Normal gland throughout. Hematoxylin and eosin. ×27.

FIG. 10. Section of inguinal gland of Rabbit R 1160 after 45 daily intravenous injections of chick embryo extract, showing an increase in size and number of secondary nodules and an increase in internodular lymphatic tissue. Compare size and histology of this gland with that of Fig. 9 and note cellular response in the former. Hematoxylin and eosin. ×27.

FIG. 11. Section of inguinal gland of Rabbit R 1158 after daily subcutaneous injections of chick embryo extract. The very hyperplastic gland consisted largely of diffusely distributed lymphatic tissue. This was rich in lymphoblasts with heavily basophilic cytoplasm. Hematoxylin and eosin. ×27.

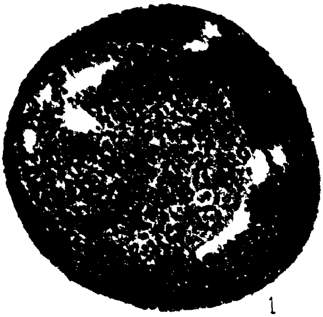
PLATE 27

FIG. 12. Section of spleen of control Rabbit R 1186 after 37 daily intravenous injections of normal salt solution. No evidence of hyperplasia. Hematoxylin and eosin. ×27.

FIG. 13. Section of spleen of Rabbit R 1157 after 19 daily intravenous injections of chick embryo extract. Note the increase in size and number of Malpighian bodies as compared with control spleen of Fig. 12. Hematoxylin and eosin. ×27.

FIG. 14. Section of spleen of Rabbit R 1163 through germinal center. This animal received 25 daily intravenous injections of a solution of egg albumen. Note increase in number of mitotic figures and large cells with basophilic cytoplasm, typical of the spleens from all rabbits receiving these injections. Hematoxylin and eosin. Oil immersion.

FIG. 15. Section of spleen of Rabbit R 1160 through germinal center. This animal received 45 daily intravenous injections of chick embryo extract. Note evidences of hyperplasia as in Fig. 14. Hematoxylin and eosin. Oil immersion.



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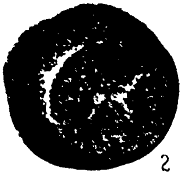

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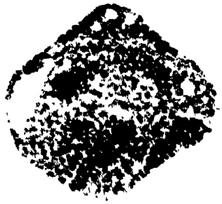

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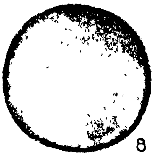

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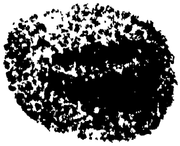

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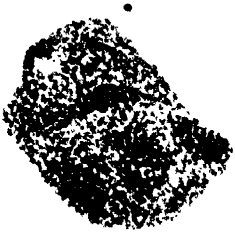

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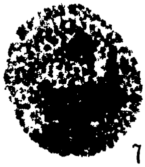

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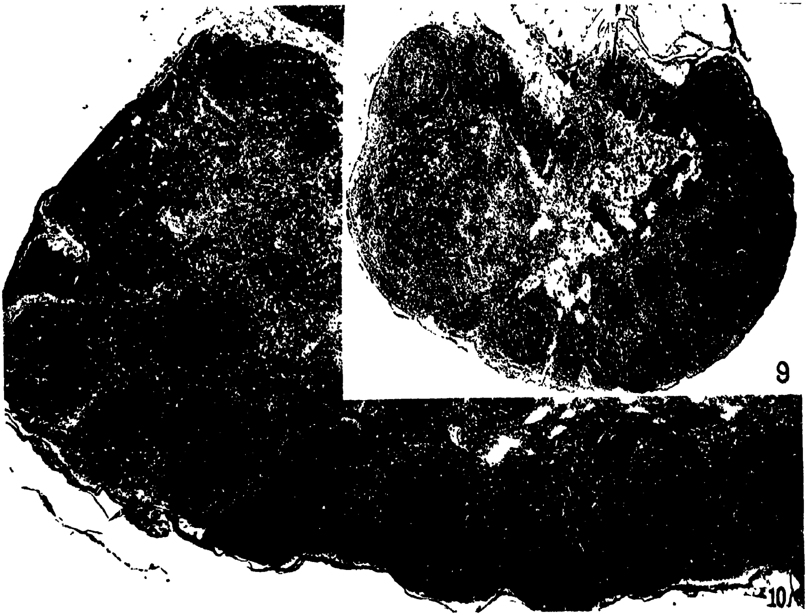
(Wiseman: Age of lymphocytes in peripheral blood)



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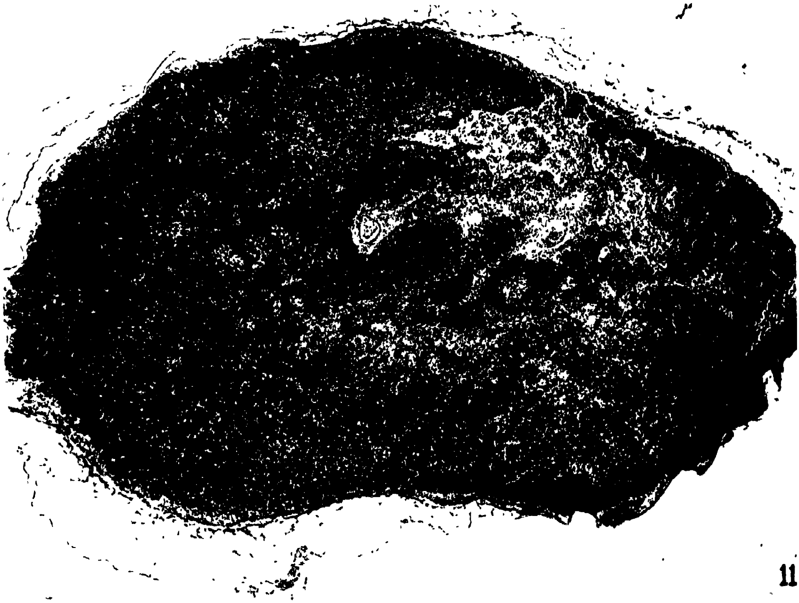

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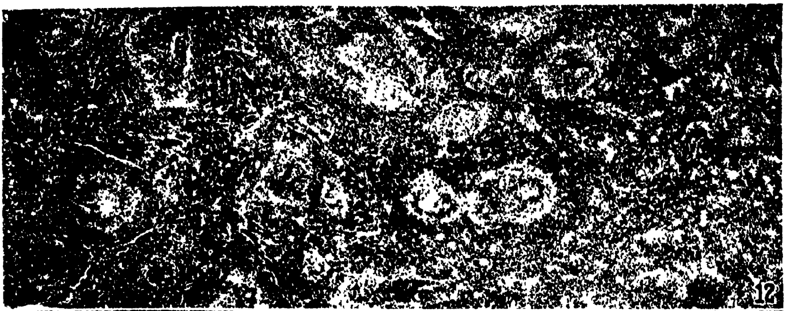
Photographed by Louis Schmidt

(Wiseman: Age of lymphocytes in peripheral blood)



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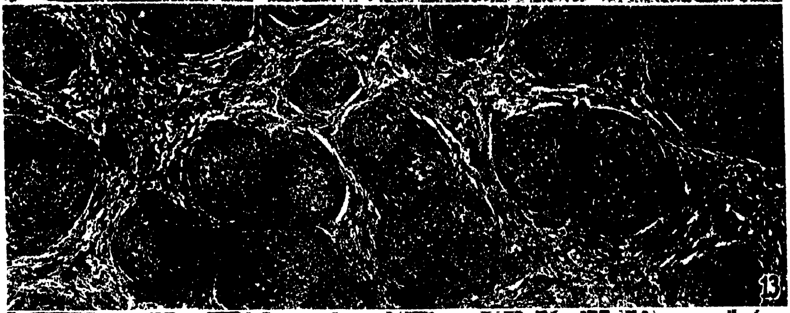

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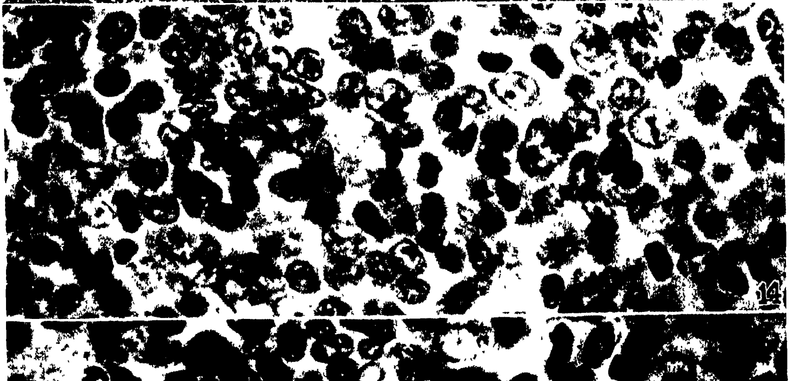

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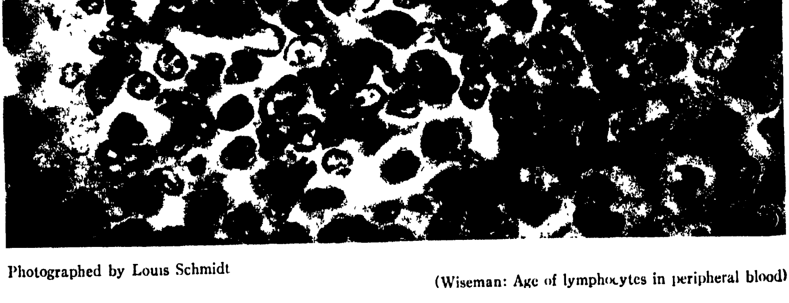

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(Wiseman: Age of lymphocytes in peripheral blood)

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SPONTANEOUS AND EXPERIMENTAL INFECTION OF PIGEONS WITH B. AERTRYCKE

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INTRODUCTION

During the course of some experiments upon blood formation in pigeons, which had first been undernourished for varying periods of time and then maintained upon diets consisting mainly of fetal beef tissues, an occasional bird became critically ill, developing diarrhea, grave anemia, and high leucocytosis with many young myeloid cells in the peripheral blood. Death invariably occurred within a few days after the onset of these symptoms. Such pigeons, though greatly emaciated, showed at autopsy marked enlargement of the liver, spleen and kidneys, as well as extreme myeloid hyperplasia of the bone marrow. Throughout the liver and kidneys, innumerable small yellowish gray points and irregularly shaped zones were grossly visible. Upon microscopic examination these areas were found to consist mainly of large numbers of myelocytes grouped about the blood vessels in these organs. The spleen showed a marked increase of large, clear, mononuclear, phagocytic cells and almost complete absence of lymphoid tissue, but contained no such accumulations of myelocytes as were seen in the liver and kidneys.

These changes presented a striking contrast to the fatty bone marrow and atrophied organs of the other equally poorly nourished pigeons of our series dying or killed after identical experimental procedures. Though it appeared that the exceptional pigeons were dying of some unusual complication, possibly of bacterial origin, we were unfamiliar with any variety of microörganism which had been shown to be capable of producing such lesions. Therefore, the changes in the blood and bone marrow, as well as the extensive extramedullary formation of myeloid tissue, seemed to us to present a problem worthy of further study.

PRELIMINARY EXPERIMENTS

No bacteriological studies were made upon the first three birds exhibiting the unusual features just mentioned, but cultures of the blood, liver, spleen, kidney and bone marrow of the fourth bird (Pigeon 86) to die under these conditions all gave pure growths of a small bacillus which was identified by Dr. L. T. Webster of The Rockefeller Institute as *Bacterium aertrycke.*

This organism, which varies from 1 to 2.5 microns in length and from 0.3 to 0.4 of a micron in thickness, is a Gram-negative, motile rod with rounded ends. In smears prepared from young cultures, the bacillus varies in morphology from rod to almost coccoid forms and its body stains evenly, though individual organisms with more deeply staining ends are usually present. Numerous long, peritrichal flagella are revealed by appropriate stains. In cultures, both acid and gas are produced in dextrose, maltose, mannite, levulose, xylose, galactose and dulcite, the reactions being more marked in the last four sugars than in the three former ones. Saccharose and lactose are not affected, even after several days' incubation. Indol is not formed. Litmus milk is rendered slightly acid within the first twenty-four hours but the reaction later changes to alkaline. Milk is not clotted. On potato the growth is very poor, forming only a faintly visible, grayish white pellicle. Coagulated serum and gelatin are not liquefied. In broth the growth produces even cloudiness, but after several days a grayish white sediment is usually formed. Cultures on lead acetate medium show that H_2S is produced. On meat infusion agar slant the organism forms a thin, transparent, confluent growth, a characteristic of all bacteria belonging to the paratyphoid group. On blood agar plates the growth is luxuriant; large, round, flat colonies with pale centers are formed in twenty-four hours. On China blue rosolic acid medium the colonies are small, round, slightly elevated and of distinctly pinkish color.

The immunological behavior of this organism strongly suggests that it represents a separate species. Cultures are agglutinated by immune pigeon and rabbit sera in high dilutions, the usual titre being

1:2560. *B. typhosus* and *B. paratyphosus B* are agglutinated by anti-aertrycke serum in dilutions of 1:40 and 1:10 respectively. Rabbit sera rendered immune to *B. typhosus*, *B. paratyphosus A*, *B*, and *C* usually agglutinate *B. aertrycke* in dilutions up to 1:40. The blood of normal pigeons rarely contains agglutinins for *B. aertrycke*, and even on these occasions the bacilli are not agglutinated in dilutions greater than 1:20.

Inasmuch as we have subsequently used the culture of organisms isolated from the liver of Pigeon 86 to produce all experimental infections, we submit the following complete protocol of this bird.

TABLE I

Protocol of Pigeon 86

Diet	Full grain (Apr. 24-26)	Starvation (Apr. 27-May 3)	5 gm. fetal liver daily (May 4-16)					Food refused	
Dates blood examined	Apr. 25	May 2	May 6	7	10	13	14	15	16
White blood cells (thousands per cmm.)	14	4	11	9	14	55	69	133	187
Granulocytes %	40	54	78	86	64	87	88	91	85
Myelocytes %	0	0	0	0	0	0	0	0	5
Lymphocytes %	42	44	12	4	26	1	5	4	4
Monocytes %	18	2	10	10	10	12	7	5	6
Red blood cells (millions per cmm.)	3.7	2.6	3.3	3.1	3.1	2.5	2.2	1.9	1.2
Thrombocytes (thousands per cmm.)	75	16	25	36	37	26	59	33	37
Weight (gm.)	425	355	320	320	300	280	265	245	230 Died

The *liver* and *kidneys* were found to be greatly swollen and studded with massive accumulations of myelocytes intermingled with smaller, basophilic, poorly differentiated, mononuclear cells. Many large mononuclear phagocytes and bacteria were found in the sinuses of the liver. The *spleen* was also much enlarged, due mainly to the presence of large, mononuclear phagocytes, but showed no such accumulations of myeloid cells as were seen in the liver and kidneys. The heart, lungs, alimentary tract, reproductive organs and brain were unaltered.

At the time of autopsy the blood as well as emulsions of the various organs of Pigeon 86 were injected into a series of normal birds. Of three pigeons, each injected intraperitoneally with 1 cc. of blood, none developed any symptoms of disease. When these birds were killed for study a month later, no anatomical changes were found, but *B. aertrycke* was grown from the livers of two of them. Of seven pigeons injected intraperitoneally with 2 cc. of a thick saline emulsion of liver, kidney, or bone marrow, two which had received liver became acutely ill within twenty-four hours and died forty-eight hours and five days respectively after inoculation. Shortly before death one of these birds exhibited a leucocytosis of 75,000 cells per cmm., of which 3 per cent were myelocytes. The other showed only a slight increase in total number of leucocytes, 21,000 per cmm., but of this number 71 per cent were myelocytes. These two pigeons showed acute fibrinopurulent peritonitis, as well as the same types of anatomical changes seen in Pigeon 86, and *B. aertrycke* was recovered from the liver of each of them in pure culture. None of the remaining five birds showed any symptoms of disease following injection, or any macroscopic lesions* when killed a month later, but *B. aertrycke* was grown from the livers of three of them at this time. Cultures made from the livers of the other two birds were sterile.

Three normal pigeons were injected intraperitoneally with 0.1 cc., 0.5 cc., and 1 cc. respectively of a pure 24 hour broth culture of *B. aertrycke*, originally isolated from the liver of Pigeon 86. The first bird showed no manifestations of disease and no anatomical changes when killed a month later. The other two birds, however, died four days and thirty-six hours respectively after inoculation, showing all of the clinical and most of the anatomical characteristics of the disease under study. In addition, fibrinopurulent peritonitis was present in both instances. No myelocytic infiltrations were found in the liver and kidneys of the bird dying thirty-six hours after inoculation. From the livers of all three pigeons *B. aertrycke* was isolated in pure culture.

It therefore seemed highly probable that infection with *B. aertrycke* was the cause of the unusual lesions and death of certain of our birds but, inasmuch as each pigeon was kept in a separate cage and cultures of their water and food failed to reveal the presence of this organism, the source of the infection was not clear. However, since it had been demonstrated experimentally that small doses of *B. aertrycke* had little or no effect upon normal pigeons, and, moreover, that these bacteria often survived within the body for at least a month without producing symptoms of disease or anatomical changes, it did not appear unlikely that certain pigeons naturally harbored minimal numbers of this organism and developed generalized infection when subjected to a state of malnutrition. At this point it is of interest to note that a single previously normal pigeon (No. 94), after a period of eight days' starvation, became unduly ill, totally refused food and died within forty-eight hours, showing all of the lesions accompanying infection with *B. aertrycke*. This organism was recovered in pure culture from the liver.

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* In the removal of this work from New York to Peiping the microscopic preparations made from the tissues of these birds were unfortunately lost.

In order to determine whether or not pigeons dying of malnutrition, but showing no evidence of bacterial infection, ever harbored *B. aertrycke* in their tissues, cultures were made of the livers of twenty such birds which had been kept under experimental conditions identical with those of the pigeons dying of infection with this organism. Sixteen of these cultures showed no growth of bacteria, but from four birds *B. aertrycke* was recovered. Though none of these four pigeons showed changes in the blood before death which would suggest infection with *B. aertrycke*, in two of them the characteristic lesions accompanying infection with this organism were found. The other two showed only the effects of malnutrition.

The results of these preliminary studies may be summarized as follows: In a series of fifty-two pigeons, which first had been subjected to a period of starvation and then maintained for varying periods of time on fetal beef tissues, seven cases of an acute, fatal disease associated with marked anemia and enormous activity of myeloid cells occurred. From three of these birds, which were studied bacteriologically, *B. aertrycke* was isolated in pure culture, and in the tissues of the other four an abundance of bacteria morphologically identical with this organism were demonstrated. Four additional instances of the presence of *B. aertrycke* were encountered in twenty routine bacteriological examinations of pigeons of this series showing no clinical evidence of bacterial infection. Two of these showed anatomical changes attributable to the infection with *B. aertrycke*, while the other two did not.

Although it was demonstrated that a disease process could be initiated in normal pigeons by inoculation of tissue from an infected bird as well as by inoculation of cultures of *B. aertrycke*, the nature of the lesions, in both the spontaneous and experimental infections, caused us to have some doubt as to their exclusive relationship to this organism. The lesions in which the bacteria were found, consisting of aggregations of mononuclear phagocytes and polymorphonuclear leucocytes, were readily explained. However, such massive myeloid hyperplasia of the bone marrow, the frequent appearance of large numbers of myelocytes in the peripheral blood, and the striking heterotopic formation of myeloid tissue in the liver and kidneys presented a picture of myeloid activity hitherto unrecorded in the course of bacterial infections. Furthermore, since *B. aertrycke*, in several instances, had been found in pigeons which showed no symptoms or anatomical evidence of active infection with this organism, and also had been shown experimentally to survive in the tissues of pigeons for considerable periods of time without producing disease, it was obvious that further facts must be sought before the relationship of this organism and the interesting lesions with which it was frequently associated in pigeons could be understood.

Methods

Each bird, upon entering the laboratory, was placed in a separate cage and observed for a period of two weeks, during which time the weight was followed and the blood examined several times. Only those pigeons which appeared normal during this time were used for experimentation.

Supravital staining with neutral red and Janus green,[1] which facilitates a ready distinction of all the cellular elements of the blood, was used routinely in making the differential cell counts. Striking changes in the blood, such as the appearance of numerous young myeloid forms, were always checked in fixed films. The total counts of the red and white cells were made according to the method of Forkner,[2] which we have found to be thoroughly reliable.

In order that experimental infection might be more nearly comparable to infection naturally acquired, we have always introduced the bacteria by mouth, slowly feeding 1 cc. of a 24 hour pure broth culture, drop by drop, with a small pipette. After infection, the blood was studied at frequent intervals until death or recovery. Autopsies, as well as bacteriological and serological studies, were done promptly after death, an occasional moribund bird being killed in order to secure perfectly fresh tissues.

To test our cultures for the presence of some filterable form of microörganism or other substance which might have been responsible for the excessive growth of myeloid tissue regularly accompanying infection with *B. aertrycke*, normal pigeons were fed or infected with sterile filtrates prepared from this organism.

To serve as controls for our other observations, bacteriological, serological and anatomical studies were made upon a series of apparently normal pigeons.

The Blood of Normal Pigeons

The blood of normal pigeons has been the object of study of only a few investigators,[3,4,5] but all of those who have dealt with it have reported marked fluctuations in number of all types of blood cells.

During the course of our experience, based upon 182 apparently healthy pigeons bought at random in the markets of New York and Peiping, we have found that the red blood cells remain unusually constant in individual birds, fluctuations of as much as 400,000 cells being quite exceptional. When first brought into the laboratory, most pigeons show erythrocyte counts of 3,500,000 to 4,000,000 cells per cmm. Only an occasional bird shows a slightly higher count, while erythrocyte counts lower than 3,000,000 almost invariably have been found to be associated with some obvious disease.

The white blood cells, however, have shown considerably greater fluctuations in number than have the erythrocytes. Though the total number of white cells was generally found to lie between 8,000 and 20,000 per cmm., an occasional bird, in which no disease was apparent, was studied which presented a count far above or below these levels. We have seen a few such pigeons with leucocyte counts of 50,000 cells per cmm., for which there was no obvious explanation.

However, after being kept in the laboratory for a few days, the white cells in such instances have invariably fallen to normal levels and later on have shown no tendency to rise to their original height.

The percentages of the different varieties of blood cells also have been found to vary greatly in different birds. They have remained quite constant in individuals, though the total white cell counts of these same pigeons have varied as much as 10,000 cells from day to day. White counts made in rapid succession upon the same bird almost always agree closely.

The morphological characteristics of the various elements of the pigeon's blood, as far as we know, are nowhere adequately illustrated, *though Forkner[2] has recently presented a well illustrated study of the blood of normal fowls, which in most essential features resembles that of the pigeon. We have, therefore, devoted most of the accompanying Plate 67, which illustrates supravitally stained cells from the blood of pigeons, to the early myelocytic types encountered in the circulation in our experimental birds.*

The granules of the main group of polymorphonuclear leucocytes (Figs. 1 and 2) of the pigeon, as in the fowl, are rod-shaped but exhibit a decidedly more brilliant and darker yellowish red color when stained either supravitally with neutral red or in fixed films with Wright's stain. We have found no cells in the pigeon's blood closely corresponding to the "pseudoeosinophils" found in small numbers in the fowl's blood by Forkner, but we have observed regularly a few myeloid cells, not present in the blood of fowls, with pleomorphic or round nuclei and large, brilliantly eosinophilic granules in their cytoplasm. Such cells, which generally exist only in small numbers, less than 5 per cent, are found to be much more numerous in occasional birds, sometimes comprising 50 per cent of the total number of leucocytes. The relationship of these cells to the other cells of the myeloid series is not clear. They apparently tend to remain high in individual birds which show no other abnormality but, if for any reason a leucocytosis occurs, their numbers have never been observed to increase. We have looked upon such cells as true eosinophils and at first thought their large numbers were perhaps associated with parasitic infections. Though this association has existed in a few instances, such is frequently not the case. Only recently we have seen an appar-

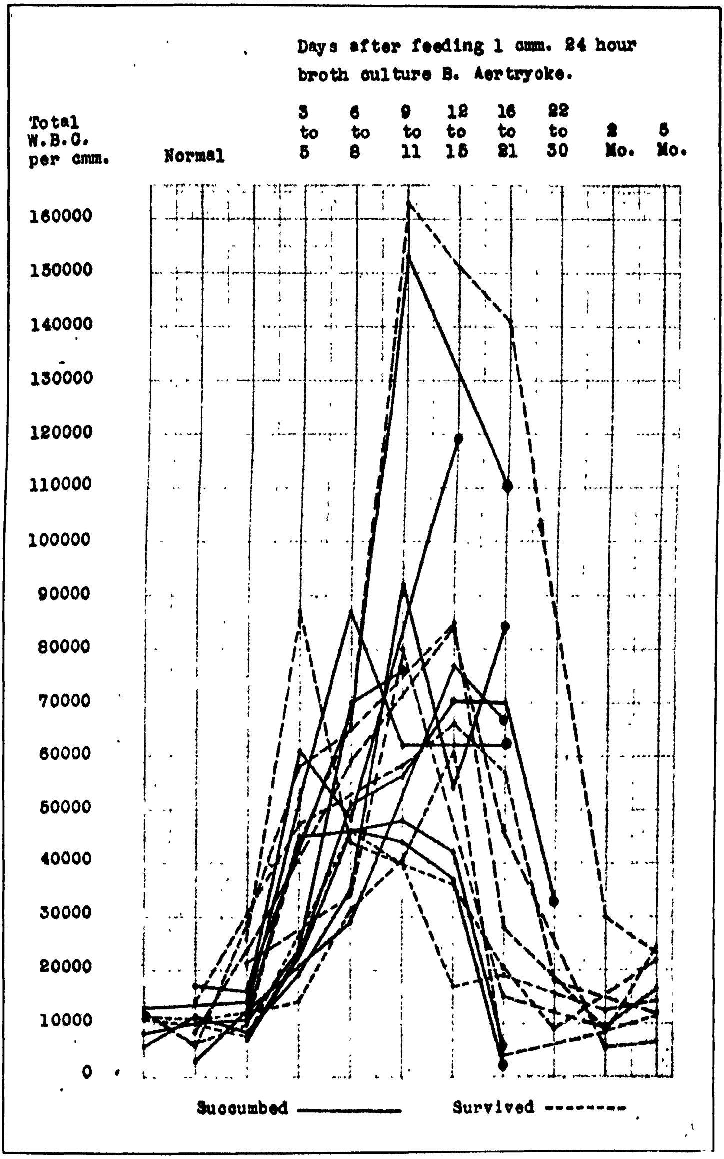
Days after feeding 1 cmm. 24 hour
broth culture B. Aertryoke.
Total
W.B.C.
per cmm.
Normal
3 to 5
6 to 8
9 to 11
12 to 15
16 to 21
22 to 30
2 Mo.
5 Mo.
160000
150000
140000
130000
120000
110000
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
Succumbed ————
Survived --------

CHART 1

ently normal pigeon with the duodenum and ileum greatly distended with a solid mass of small nematodes in which the total white cell count was 15,000 per cmm., of which only 2 per cent were eosinophilic myeloid cells with round granules. The granules of the basophils of the pigeon are very small, round and stain deep crimson with neutral red. The monocytes (Figs. 12, 13), lymphocytes (Fig. 14), erythrocytes, and thrombocytes show only very slight variations in morphology from those of the fowl. The general appearance of the myelocytes of the two species is about the same, though slight differences in color of the granules exist in both supravitally stained (Figs. 5 to 11) and fixed preparations.

In the following table are summarized the results of our studies of the blood of normal pigeons. Only birds which were apparently free from disease have been included. The figures given represent average values.

TABLE II

Blood Counts on Normal Pigeons

	New York	Peiping	Total
Number of Pigeons	100	82	182
Number of Blood Counts	238	139	377

	New York		Peiping	
	Total cells per cmm.	Percentage	Total cells per cmm	Percentage
Erythrocytes	3,813,000	..	3,803,000	..
Leucocytes	13,100	..	15,200	..
Thrombocytes	34,060	..	42,520	..
Eosinophilic leucocytes with rods	5,371	41	5,320	35
Eosinophilic leucocytes with round granules	917	7	760	5
Basophilic leucocytes	393	3	304	2
Lymphocytes	5,109	39	7,144	47
Monocytes	1,310	10	1,672	11

The Experimental Infection of Pigeons with B. Aertrycke

After two weeks preliminary observation, during which time their blood was studied, seventeen apparently normal pigeons were each fed 1 cc. of a 24 hour broth culture of *B. aertrycke*. All of these pigeons became acutely ill within thirty-six hours, most of them developing diarrhea and showing loss of weight, although given food and water in unlimited amounts. Nine of them died within ten to twenty-five days after infection; the remaining eight recovered.

Changes in the Blood: After feeding *B. aertrycke* the total number of leucocytes, which had varied in different birds under normal conditions from 6,000 to 29,000 cells per cmm., rose sharply to levels of 46,000 to 160,000 cells per cmm. (Chart 1.) Most of the pigeons which died succumbed at a point near the peak of their leucocytosis, but in two birds, after only moderately high leucocytoses of 46,000 and 48,000 cells per cmm., the total number of white cells suddenly fell to 2,000 and 6,000 cells per cmm. respectively. Examination of the bone marrow of these two birds after death showed very few myeloid forms but did reveal a striking degree of hyperplasia of the erythrogenic cells. Extensive myeloid infiltration was present, however, in kidneys and liver of each bird. This particular observation is suggestive of the two types of leukemic manifestation, erythroleucosis and myeloid leucosis, reported as occurring in different fowls inoculated with identical material from an animal dying of a typical myeloid leukemia. That is, with apparently the same underlying stimulus, two different responses may be elicited, depending upon the individual bird. It is not known at present what the nature of the factors governing the individual response may be.

The leucocytosis following infection with *B. aertrycke* was clearly due primarily to an increase in number of the eosinophilic polymorphonuclear leucocytes with rod-shaped granules (Chart 2). Within three to five days after infection mitochondria became visible in the motile granulocytes. The delicately shaped rods of these cells became greatly swollen (Figs. 1, 2, 4) assuming an oval or rounded form though they still stained brilliantly, and atypical microcytes (Fig. 3) were found. After a week many of these younger adult forms of the eosinophilic rod cells, as shown by their slightly indented nuclei and numerous mitochondria, were seen regularly in the peripheral blood. The motility of all granulocytes was always strikingly decreased during the entire course of the infection. Actual myelocytes, frequently very young forms, appeared in practically all pigeons of this group in numbers varying from 2 to 10 per cent of the total number of leucocytes, but in no instance of experimental infection have

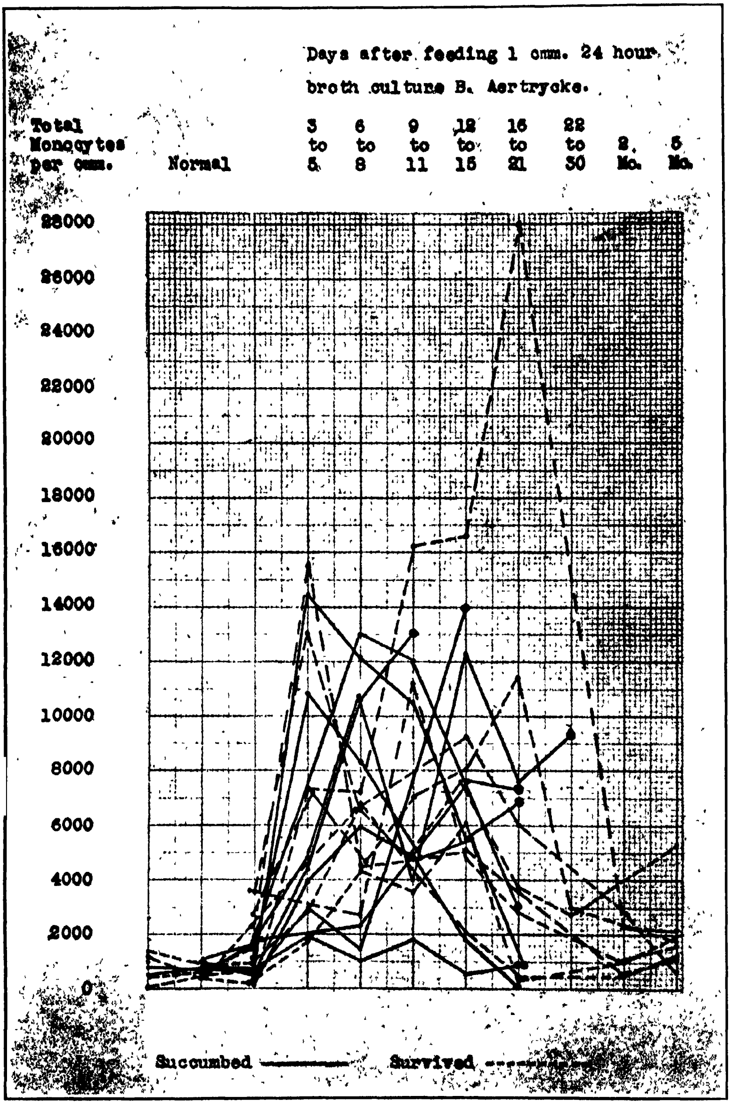
Days after feeding 1 cmm. 24 hour
broth culture B. Aertrycke.
Total
Monocytes
per cmm.
Normal
3 to 5
6 to 8
9 to 11
12 to 15
16 to 21
22 to 30
2. Mo.
5 Mo.
28000
26000
24000
22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Succumbed
Survived

CHART 2

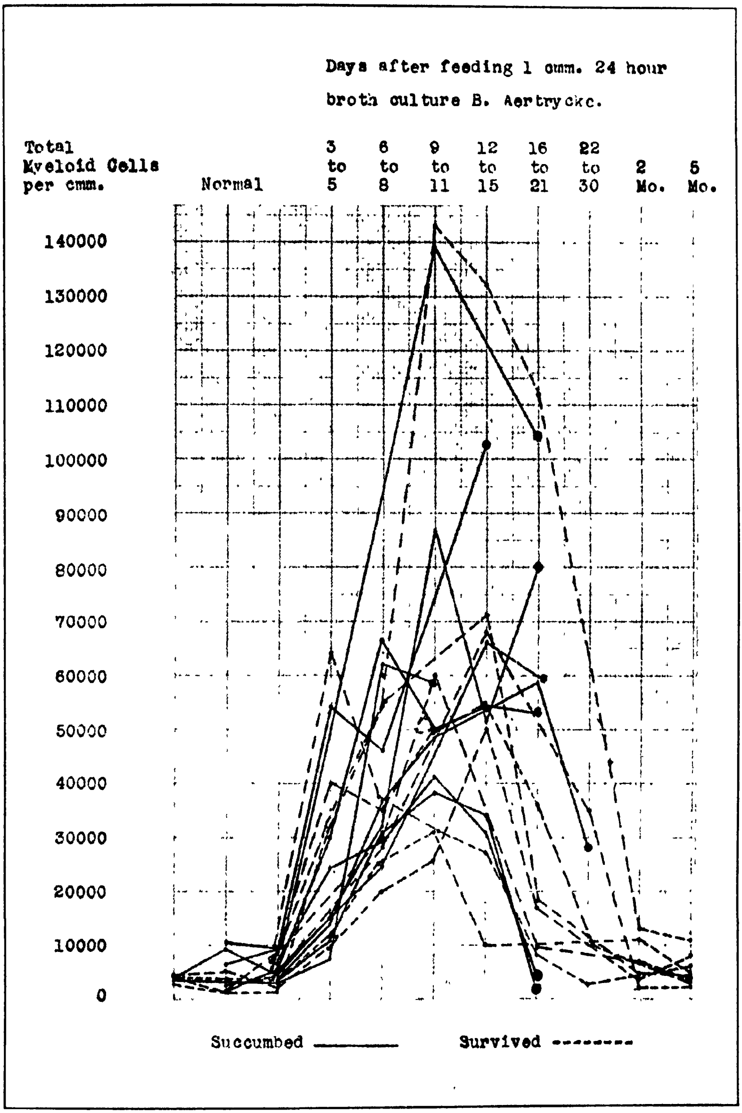
Days after feeding 1 cmm. 24 hour
broth culture B. Aertrycke.
Total
Myeloid Cells
per cmm.
Normal
3 to 5
6 to 8
9 to 11
12 to 15
16 to 21
22 to 30
2 Mo.
5 Mo.
140000
130000
120000
110000
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
Succumbed
Survived

CHART 3

we observed such a great number of myelocytes in the peripheral blood as frequently occurred in undernourished pigeons dying from infection with *B. aertrycke* naturally acquired. Fig. 7 represents the late myelocytes "C,"[32] with the complement of specific preleucocytic granules nearing the maximum. The myelocytes of the bird have spherical granules throughout the period of maturation in bone marrow, the change to rods occurring coincident with the change in staining reaction, lobing of the nucleus and the acquisition of motility, which immediately precede the extramedullary circulatory appearance and function of these cells. Under normal conditions only fully mature cells with none or very occasional mitochondria, and only rod-shaped granules appear in the circulation. Figs. 5, 8, and 10 represent the stage known as myelocyte "B," midway between myelocyte "C" and the earliest myeloid cells showing specific granulation, namely myelocytes "A" (Figs. 6, 9 and 11). No changes in the polymorphonuclear leucocytes with round eosinophilic granules or in the basophils were noted.

The monocytes showed considerable increase in total number after infection with *B. aertrycke*, tending to reach their highest point before the fifteenth day. Many young monocytes with dense, homogeneous cytoplasm and numerous mitochondria appeared regularly during the infection (Chart 3).

The lymphocytes exhibited a general tendency to fall during the course of the infection. Especially in those birds which succumbed, the diminution of lymphocytes was most marked (Chart 4).

Changes in the Viscera: The changes observed in the viscera of pigeons following oral administration of a large dose of *B. aertrycke* have been almost identical in all birds dying as the result of infection. Therefore, since individual birds showed only minor variations in the degree of lesions commonly present, these changes will be discussed collectively. By far the most interesting are those of the liver, kidney, bone marrow and spleen.

The *liver* is always enlarged, at times reaching a weight of 20 gm. It has an opaque, swollen appearance, is generally of pale, brownish red color, and throughout the organ small, round or irregularly shaped patches of opaque, yellowish gray tissue are seen. Microscopically, the liver cells are found to be greatly swollen and the sinuses filled with large, pale, phagocytic cells containing cellular débris, hemosiderin and occasionally red blood cells. Such phagocytic cells, together with a few polymorphonuclear leucocytes, are often found in solid masses, showing varying degrees of necrosis in their central areas where clumps of small Gram-negative bacilli are usually present (Figs. 15, 16). Many such bacilli are scattered in smaller number throughout the liver sinuses. In addition to these lesions, which clearly seem to be due directly to the bacteria which are found within them, each portal and hepatic vein is found to be surrounded by varying numbers of myelocytes intermingled with lesser numbers of slightly smaller, basophilic, mononuclear cells, which contain no granules in their cytoplasm (Figs. 17, 18, 19). In early infections these latter cells have occasionally outnumbered the myelocytes with which they were associated. The myeloid tissue usually present is quite excessive, frequently forming areas visible to the naked eye. Bacteria have never been found in these lesions.

The *kidneys* are always considerably swollen and of pale, yellowish brown color. Large foci of myelocytes and basophilic, mononuclear cells apparently arising about the blood vessels, are regularly present which closely correspond to the lesions seen in the liver (Fig. 20). In some instances there are also small collections of mononuclear, non-granular cells scattered throughout the interstitial tissue of the kidneys. The epithelium of the tubules is generally much swollen.

Of all the changes occurring in pigeons after infection with *B. aertrycke* those found in the *bone marrow* are perhaps the most extensive. The radius, ulna and femur, which were examined regularly, were filled with solid, firm masses of grayish red, opaque marrow, which literally burst forth from the cracks in the shaft during the process of removal. On microscopic examination one sees a solid mass of myeloid cells in which young forms predominate. The capillaries are collapsed by pressure of the proliferating myelocytes and erythrogenesis, so conspicuous in the marrow of normal pigeons, is seen only in certain very limited areas (Figs. 21, 22). Scattered throughout the marrow are found in many instances large and small aggregations of pale, mononuclear cells similar to those seen in the liver. Occasionally, bacteria may be demonstrated in these areas. Capillaries filled with fresh thrombi and bacteria are seen frequently.

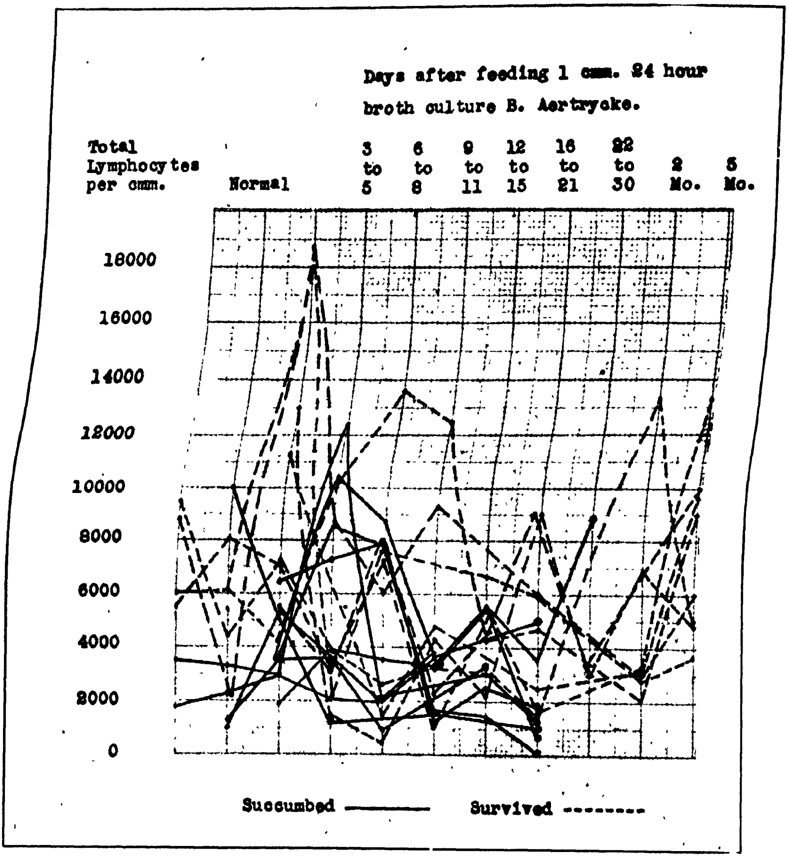
Days after feeding 1 cmm. 24 hour
broth culture B. Aertrycke.
Total
Lymphocytes
per cmm.
Normal
3 to 5
6 to 8
9 to 11
12 to 15
16 to 21
22 to 30
2 Mo.
5 Mo.
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Succumbed
Survived

CHART 4

The *spleen* is enlarged from one to three times its normal size, is of very soft consistency and pale pink or yellowish brown in color. Microscopically, the most striking alteration is found to be a marked proliferation of large, clear mononuclear cells. This change, which takes place in the follicles as well as the pulp, is attended with almost total disappearance from the entire spleen of cells which can be identified as lymphocytes. Within the sinuses and throughout the pulp many such phagocytic cells, frequently grouped in nodules with a few polymorphonuclear leucocytes about cellular débris and bacteria, are present. Though fairly numerous polymorphonuclear leucocytes and, in a few instances, occasional myelocytes are seen scattered throughout the spleen, no collections of myeloid tissue occur in the spleen which are in any way comparable to those seen regularly in the liver and kidneys. In the case of only one pigeon, which was shown to be ill with acute *B. aertrycke* infection when brought into the laboratory, were large areas of young myelocytes found in the spleen.

The *lungs*, in most instances where infection has been induced by mouth, have shown lobular pneumonia of an unusual type. Situated only in the dependent (anterior) portion of the lungs, about bronchi plugged with necrotic exudate, were caseous, often coalescing areas of consolidation which were sharply demarcated from the surrounding tissue. Microscopically, such areas, within which were myriads of Gram-negative bacilli, were found to be composed of necrotic lung tissue and exudate well encapsulated by a thick zone of mononuclear phagocytes and polymorphonuclear leucocytes. *B. aertrycke* was recovered regularly from such patches of pneumonia. The constant, characteristic distribution of these lesions strongly suggests that they were caused by aspiration of a portion of the culture of bacilli with which the birds were fed. No lesions of any kind have occurred in the lungs of pigeons naturally infected with *B. aertrycke.*

We have never found any significant lesions in the *intestine* of pigeons following natural or experimental infection with *B. aertrycke.* Upon being introduced by mouth, these organisms may be recovered from the stool during the following three or four days in about 50 per cent of the birds. After this time, however, they tend to disappear from the intestine. Intestinal contents and mucosa have been cultured in twenty-six pigeons dying from five to twenty-five days after oral administration of *B. aertrycke*, but in only six instances was the organism recovered. Pure cultures of *B. aertrycke* were grown from the liver of each of these birds and morphologically identical organisms demonstrated in sections of other organs.

The *heart*, *pancreas*, *adrenals*, *thyroid*, *ovaries*, and *testes* have shown no lesions worthy of note. We have examined the *brain* in only four birds infected with *B. aertrycke*, but no lesions were found in these instances.

Experiments with Bacteria-Free Filtrates Prepared from Cultures of B. Aertrycke

Although the oral administration to pigeons of what seemed to be pure cultures of *B. aertrycke* was followed regularly by the disease which we have described, the deduction might not follow that all of the anatomical changes present were caused by this organism. It was clear that the lesions containing bacteria were the direct result of infection with *B. aertrycke*, but the possibility still existed that our cultures were contaminated with a filterable microörganism or contained some filterable agent which was responsible for the changes in the myeloid cells. Accordingly it was decided to test upon pigeons the effect of bacteria-free filtrates prepared from virulent cultures of *B. aertrycke*.

From a 200 cc. flask of a 24 hour broth culture of *B. aertrycke*, 100 cc. was passed through a VV Berkefeld filter. To test its sterility 40 cc. of this filtrate was immediately inoculated into a fresh flask of sterile broth, and this flask, together with the flask containing the remaining 60 cc. of filtrate, incubated overnight. No growth of bacteria occurred in either of these two flasks. Each of six apparently normal pigeons, whose blood had been found free of natural agglutinins to *B. aertrycke*, was then fed 2 cc. of this sterile *B. aertrycke* filtrate. To test the potency of the organism from which the filtrate was made, 1 cc. of the original broth culture of *B. aertrycke*, then 36 hours old, was fed to each of six other normal pigeons.

As shown in Chart 5, no appreciable changes occurred within twenty-one days in the blood of the six pigeons fed with filtrate, nor did they show any loss of weight or other evidence of disease. When

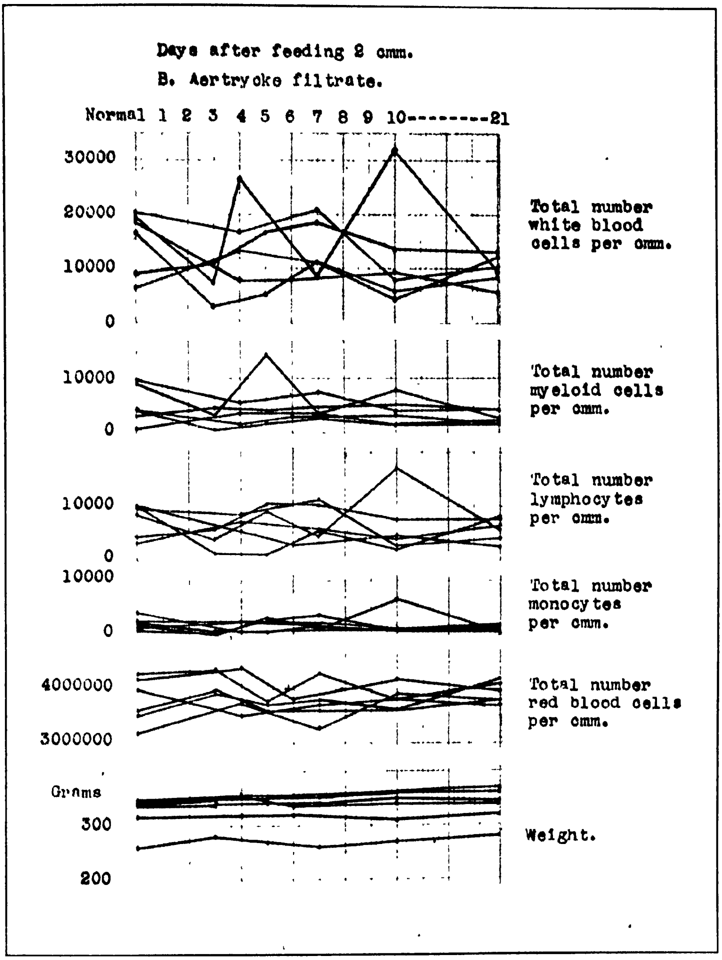
Days after feeding 2 cmm.
B. Aertryoke filtrate.
Normal 1 2 3 4 5 6 7 8 9 10--------21
30000
20000
10000
0
Total number white blood cells per cmm.
10000
0
Total number myeloid cells per cmm.
10000
0
Total number lymphocytes per cmm.
10000
0
Total number monocytes per cmm.
4000000
3000000
Total number red blood cells per cmm.
Grams
300
200
Weight.

CHART 5

they were killed at the end of this time, no agglutinins for *B. aertrycke* were found in their blood, cultures of liver were sterile in each instance, and no gross or microscopic lesions suggestive of infection with *B. aertrycke* were found in any of their tissues.

All of the six pigeons fed with the original culture of *B. aertrycke* from which the filtrate was made became acutely ill. Four died; two recovered. Autopsies upon the four which died showed all of the characteristic lesions of infection with *B. aertrycke* and this organism was recovered from the organs in each case. The sera of the two birds which recovered agglutinated cultures of *B. aertrycke* in dilutions of 1:320 and 1:2560 respectively one month after infection.

This experiment has since been repeated in a slightly different form. Another sample of filtrate, prepared as outlined above, was fed in amounts of 2 cc. to each of five normal pigeons and also injected intravenously in amounts varying from 0.2 to 1.5 cc. into each of five others. At the same time, as controls, two normal birds were injected intravenously with 1 and 2 cc. of sterile broth, respectively, and each of two others fed with 2 cc. of the same material. The virulence of the culture of *B. aertrycke* from which the filtrate was made was tested by feeding 1 cc. to each of two normal birds.

None of this series of ten pigeons, either fed or intravenously inoculated with *B. aertrycke* filtrate, or the four controls receiving sterile broth showed any change in blood picture, loss of weight or other evidence of disease during the ensuing month after administration of these substances. When killed at this time no gross or microscopic changes in any way resembling the lesions occurring in *B. aertrycke* infection were seen. Cultures of the liver made upon blood agar plates were universally sterile, and the blood in each case showed no agglutinins for *B. aertrycke.* Both of the controls, upon which the virulence of the culture of *B. aertrycke* was tested, died within ten days. They showed all of the characteristic symptoms and lesions produced by this organism, which was recovered from the livers of both birds.

Therefore, inasmuch as two different series of pigeons, in all numbering sixteen birds, either fed with or intravenously injected with large, single doses of broth filtrate prepared from virulent cultures of *B. aertrycke* have shown no clinical, anatomical, bacteriological or serological evidence of disease, it seems at least highly probable that all aspects of the disease-picture regularly occurring in pigeons after experimental infection with *B. aertrycke* are due entirely to that organism or its products. However, the possibility that a virus or other substance may be concerned in the alterations of the myeloid tissue is still to be kept in mind until the question has been studied further.

Studies upon Normal Pigeons

In order to gain some idea of the frequency with which pathological changes occur within the tissues of apparently normal pigeons, and to serve as a general control for our observations upon pigeons infected with *B. aertrycke*, we have studied sixteen birds which gave the appearance of being entirely free of disease. The results are summarized in Table III.

All of the six pigeons, which are stated to have been under observation for ten months, were kept in separate cages but in close proximity to the cages of pigeons infected with *B. aertrycke*. During this time their bloods were examined at frequent intervals but no significant changes were observed; none of them showed any loss of weight or other evidence of disease. The remaining twelve birds observed for one week before being killed for study were well nourished, took their food well, and their blood cells, upon two examinations, showed no pathological changes.

As seen in the table, cultures of the liver made upon blood agar plates were universally sterile, but the sera of two of the pigeons which had been kept for a long period of time in close proximity to birds infected with *B. aertrycke* were found to agglutinate *B. aertrycke* in dilutions of 1:64 and 1:32 respectively. No agglutinins were present in the bloods of any of the other birds. Attention is called to the frequency with which pathological changes are found in the liver and kidneys of apparently normal pigeons, also to the fact that occasional myelocytes are present in the tissues of such birds. No noteworthy changes of any kind were found in the bone marrow, lungs, spleen, testes, ovaries and intestine.

TABLE III

Summary of Pathological Changes in Sixteen Normal Pigeons Used as Controls for Pigeons Infected with B. Aertrycke

No.	Period of observation	Cultures of liver	Agglutination *B. Aertrycke*	Pathological changes in tissues
7	10 months	No growth	1:64	Moderate increase in small, round cells about blood vessels of liver and kidney. In a few instances round masses of these cells resembling germinal centers of lymph glands seen
8	10 months	No growth	0	Three small groups of adult polymorphonuclear leucocytes with an occasional myelocyte among them seen adjacent to portal veins
10	10 months	No growth	0	Small round cells (lymphocytes?) slightly increased about vessels of liver
11	10 months	No growth	1:32	Enormous increase of lymphocytes about vessels of liver. Moderate increase of such cells in kidney
12	10 months	No growth	0	No lesions
13	10 months	No growth	0	No lesions
72	1 week	No growth	0	Very slight increase of round cells about vessels of liver
73	1 week	No growth	0	Considerable increase round cells in liver. Small healing necroses in liver and also small nodules consisting of central giant cells and débris surrounded by large, clear monocytic cells and lymphocytes. No tubercle bacilli demonstrable. These lesions do not resemble those of tuberculosis
75	1 week	No growth	0	No lesions
77	1 week	No growth	0	Liver shows nodules as in Pigeon 73. Moderate hyperplasia of bone marrow
79	1 week	No growth	0	Liver shows 1 nodule as in Pigeon 73

TABLE III—*Concluded*

No.	Period of observation	Cultures of liver	Agglutination *B. Aertrycke*	Pathological changes in tissues
81	1 week	No growth	0	Moderate increase of round cells in liver, grouped about which are a few adult polymorphonuclear leucocytes and an occasional myelocyte. Many small basophilic cells seen about vessels and in interstitial tissue of kidney. Moderate hyperplasia of bone marrow
82	1 week	No growth	0	Periportal round cells of liver moderately increased. Also a few polymorphonuclear leucocytes and an occasional myelocyte seen in these areas. No increase of round cells in kidney but 1 small patch of myelocytes seen
84	1 week	No growth	0	No lesions
85	1 week	No growth	0	No lesions
88	1 week	No growth	0	No lesions

Studies upon Pigeons Surviving Experimental Infection with B. Aertrycke

Bacteriological, serological and anatomical studies have been made upon eight pigeons which survived experimental infection with *B. aertrycke* for periods of time varying from five to nine months. All of these pigeons were well nourished and showed no alterations in the peripheral blood or other manifestations of disease when killed for study. Each of these birds had, however, become clinically ill, had lost weight and shown marked increase of the myeloid elements of the blood for a period shortly after oral administration of *B. aertrycke*.

Bacteriological cultures made routinely from the liver on blood agar plates were universally sterile, and cultures of the intestinal contents and mucosa failed to show pathogenic organisms of any kind.

The results of the serological studies are shown in Table IV.

The changes found in the tissues of these pigeons, though of con-

siderable extent, cannot be fully understood until the histogenesis of the lesions produced by B. aertrycke has been studied further. Briefly stated, they consisted almost entirely of the healing of the destructive lesions caused by the bacteria. Pigeon 15, killed nine months after infection, showed only one small, encapsulated, caseous area in the lung, the remains of a small patch of pneumonia, no changes of any kind being present in any of the other tissues. The other seven pigeons, killed five months after infection, showed many large phagocytic cells in the liver and spleen loaded with iron pigment, numerous healing necroses in the liver, and many circumscribed caseous areas in the lungs, each surrounded by a wall of giant cells and connective tissue.

TABLE IV

Agglutination Titer of Blood Serum to B. Aertrycke

No.	Before infection	After infection		
		1 month	5 months	9 months
15	0	..	1:1280	0
16	0	0	1:160	..
20	0	1:2560	0	..
22	0	1:80	0	..
25	0	1:1260	0	..
36	0	0	0	..
38	0	1:320	0	..
42	0	1:320	1:80	..

These latter lesions, obviously the result of the characteristic lobular pneumonia produced by *B. aertrycke*, varied from microscopic size to as much as 7 mm. in diameter. The smaller ones were identical with the larger healing necroses found in the liver and also bore close resemblance to the nodular lesions frequently found in the liver of apparently normal pigeons. There was almost no trace left of the myeloid hyperplasia which may have existed during the phase of acute disease in this series of pigeons. In three birds, quite numerous small groups of adult polymorphonuclear leucocytes were seen about the blood vessels in the liver. The bone marrow had returned to its normal appearance in six of the pigeons, but in the two remaining birds gelatinous degeneration of the marrow of the radius was present.

DISCUSSION

A number of previous workers have already called attention to unusually high leucocytoses varying from 200,000 to 600,000 cells per cmm. which characteristically occur in birds during the course of certain bacterial infections. Among these may be mentioned Smith and Moore,[6] who described a disease of fowls caused by an organism to which they gave the name *B. sanguinarium*, Burckhardt,[7] and Hirschfeld and Jacoby,[8, 9] who studied fowls infected with avian tuberculosis. So great was the number of myeloid cells in the blood that Smith and Moore considered the disease which they studied to be a form of myeloid leukemia, while Burckhardt expressed the view that myeloid leukemia in fowls was a manifestation of tuberculosis. Hirschfeld and Jacoby oppose Burckhardt's opinion. We can find no blood studies upon pigeons infected with tuberculosis but have ourselves observed a pigeon dying of naturally acquired tuberculous infection whose blood contained 185,000 leucocytes per cmm., of which 92 per cent were mature polymorphonuclears. Cultures of the blood and organs of this bird on blood agar plates and broth were sterile. Though the bone marrow showed marked myeloid hyperplasia, the cells present were mostly adult leucocytes or myelocytes well advanced toward maturity. No myelocytes were seen in the blood and there was no infiltration of other organs with myeloid cells. The mouth, pharynx and bones of the paranasal sinuses showed large caseous lesions, while the liver and spleen contained countless small tubercles. In all of these lesions masses of acid-fast bacilli were present.

None of the observers who have studied these hyperleucocytoses of birds caused by bacteria has reported the presence of an appreciable number of immature white cells in the peripheral blood or the occurrence of infiltrations of such cells in the viscera. We therefore agree with Opie,[10] who recently expressed the view that those investigators reporting the experimental production of diseases closely related to leukemia by means of bacterial infection have failed to justify their claim.

In the case of both natural and experimental infection of pigeons with *B. aertrycke*, however, the situation seems to us to be somewhat different from that of other bacterial infections of birds hitherto re-

ported, though we are by no means prepared to state that this disease is in any way *fundamentally* related to myeloid leukemia of birds, mammals or human beings. At the present stage of our studies of *B. aertrycke* infection in pigeons we can call attention only to the common occurrence of large numbers of immature myeloid cells in the peripheral blood and the constant appearance of extensive myeloid foci in the liver and kidneys of birds infected with this organism. These changes, to our knowledge, have not been observed to occur in other bacterial infections. We wish to emphasize the fact that, in addition to the hyperplasia and heterotopia of the myeloid tissue here described, in which no bacteria were found, other widely spread lesions of a totally different character, consisting of accumulations of mononuclear phagocytic cells, containing *B. aertrycke* in abundance, occurred with regularity.

Inasmuch as we have not yet had the opportunity of studying fowl leukemia we can make no definite statement concerning the relationship of this disease to *B. aertrycke* infection in pigeons, though one would be inclined to consider the two conditions as separate entities. Most of our knowledge of fowl leukemia is derived from the studies of Ellermann and Bang,[11] Ellermann,[12, 13, 14, 15] Hirschfeld and Jacoby,[8, 9] and Schmeisser,[16, 17] who, in general, agree that the disease is caused by a filterable microörganism. Ellerman and Bang, who first advanced this theory, did so upon the ground that they were able to transmit the disease to a certain percentage of normal fowls by the injection of bacteria-free filtrates prepared from the organs of a fowl dying of the disease spontaneously acquired. These authors, however, do not appear to have searched very thoroughly for bacteria. No mention is made of cultures made directly from the organs of the birds spontaneously or experimentally infected, nor were the tissues of these birds examined microscopically for bacteria. The only proof offered for the sterility of the filtrate with which the disease was experimentally produced is the statement that it was clear and gave rise to no growth when cultured in several types of media. The amount of filtrate cultured was not mentioned, but it was stated that cultures of the emulsion of tissues from which the infectious filtrate was made gave a rich growth of bacteria. No further study was made of these organisms. Though it is possible that the filtrate with which Ellermann and Bang claimed to have produced leukemia in fowls was actually free of bacteria, one does not feel convinced that such was the case from the account of their experiments. Hirschfeld and Jacoby,[9] who attempted to repeat the experiments of Ellermann and Bang,[11] began their studies with a leukemic fowl obtained directly from the latter workers and were apparently successful in transmitting the disease to other fowls by injections of whole organ emulsion prepared from the diseased bird. However, their stock of fowls proved to be heavily infected with spontaneously acquired tuberculosis. The results of their experiments are not altogether clear, though they studied great numbers of birds in their attempt to separate the effects of these two diseases. It is worthy of note that Hirschfeld and Jacoby did not succeed in producing leukemia in any of their fowls by the injection of bacteria-free filtrates made from the organs of leukemic birds. Schmeisser,[16, 17] has studied a disease of fowls considered to be myeloid leukemia, which he found to be transmissible to normal birds by injection of organ emulsions made from both spontaneously and experimentally infected birds. He has written a good description of the changes in the blood and other organs but in neither of his papers has he mentioned any bacteriological studies whatever.

Just as this paper was completed the publications of Furth and Stubbs,[33, 34] appeared, confirming once more the filterable nature of the etiological agent in fowl leukemia. While the similarity is striking in certain phases of the hemopoietic response, it would seem that entirely different causative agents were at work in fowl leukemia and *B. aertrycke* infection in pigeons. Though all of these investigators who have studied fowl leukemia have described lesions apparently identical with the changes in the myeloid tissue occurring in pigeons infected with *B. aertrycke*, none has reported the presence of the widely spread collections of monocytic cells frequently grouped about bacteria which we have regularly observed. It seems unlikely that such lesions would be overlooked. Furthermore, whereas the incubation period of fowl leukemia is stated to be approximately two months, our pigeons infected with large doses of *B. aertrycke* became ill within two days, and small doses of these bacteria produced no symptoms of disease at all. In relation to this latter point may be mentioned the work of Winternitz and Schmeisser,[18] who studied *a series of fowls experimentally infected with B. sanguinarium. As previously found by Smith and Moore, their birds developed high* leucocytoses but showed no extramedullary accumulations of myelocytes. A single bird, however, developed changes in the blood characteristic of myeloid leukemia several weeks after injection of the bacteria and was found to show extensive infiltration of the liver and kidneys with myelocytes. No bacteria of any kind were recovered from this bird and these investigators were inclined to conclude that the case was one of true fowl leukemia. They suggested the possibility that leukemia may be produced in the fowl by graded doses of *B. sanguinarium*, but did not pursue the matter further.

Aside from its possible relationship to fowl leukemia, the question of *B. aertrycke* infection in pigeons is, in itself, a matter of considerable interest. Our observations, though inadequate as absolute proof, would point strongly to the assumption that pigeons frequently harbor minimal numbers of this organism which, under ordinary conditions, cause them no harm. However, under adverse conditions, such as severe malnutrition, it would seem that these bacteria might multiply and frequently become sufficiently pathogenic to produce disease.

That *B. aertrycke* infection is a natural disease of pigeons is shown by observations recently made upon a bird noted to be seriously ill when brought into the laboratory. Immediate examination of the blood showed 2,900,000 erythrocytes and 108,000 leucocytes per cmm., eosinophilic leucocytes 64 per cent, myelocytes 2 per cent, lymphocytes 20 per cent, and monocytes 14 per cent. A culture of the blood made at the same time was positive for *B. aertrycke*, which was identical in all of its characteristics with the strains isolated from spontaneous cases of *B. aertrycke* infection developing in pigeons in the laboratory in New York. On the following day a second blood culture was also positive for *B. aertrycke*. This pigeon was isolated from the other birds and observed for eight days, during which time it remained critically ill, continued to lose weight, and the granulocytes of the blood increased to 83 per cent. After death on the eighth day, all of the lesions characteristic of *B. aertrycke* infection were found in advanced degree and the organism was recovered from the liver, kidney, bone marrow and blood. Intraperitoneal injection of saline emulsion made from the liver, as well as pure cultures of the bacteria, produced both symptoms and lesions in normal pigeons identical with those occurring after experimental infection with other strains of *B. aertrycke*.

We have not yet studied the effect of oral administration of *B. aertrycke* upon animals other than pigeons, but have found these organisms to be highly pathogenic for chickens, mice, guinea pigs and rabbits when injected in fairly large doses. The animals died of septicemia within a few days but did not show myeloid alterations in their organs such as those seen in pigeons infected by the oral route.

Though *B. aertrycke* is generally mentioned in the literature in association with food poisoning in man, it also has been observed to cause disease in animals. The system of nomenclature for the group of bacteria related to *B. aertrycke* is so inaccurate, however, that relatively few of the investigators who have reported such observations appear to have been dealing with the same organism. Otte[19] has reviewed the many studies upon natural and experimental infection of birds with bacteria of this type, and Beck and Meyer[20] have studied a disease of pigeons, widespread in Germany, which was caused by a bacillus considered to be *B. aertrycke*. No blood studies were made by any of these observers. Infiltrations with mononuclear cells, similar to those we have observed in pigeons, were of common occurrence but no changes in the myeloid cells were reported.

Beaudette and Edwards[21] have reported an epidemic in canaries caused by this organism, Beaudette[22] an epidemic in young pigeons, and Doyle[23] has described an epidemic among young chicks. Meyer and Matsumura[24] state that a considerable percentage of wild rats are carriers of *B. aertrycke*. Petrie and O'Brien[25] described an epidemic in guinea pigs which they thought was due to a filterable microörganism, but regularly cultured *B. aertrycke* from the dead animals. This organism was highly pathogenic for normal guinea pigs when injected subcutaneously, but of low virulence when administered by mouth. Topley and Ayrton,[26] working with white mice, found the greatest variation in the behavior of different strains of *B. aertrycke*. Some strains were excreted regularly from the intestine after oral administration, while others appeared only transiently, in some instances fatal infection taking place without the organisms ever appearing in the feces. These workers also showed that *B. aertrycke* frequently remained in the tissues of mice without causing disease. Topley,[27] at a later date, demonstrated *B. aertrycke* of unaltered virulence in the spleens of mice actively immunized to lethal doses of this organism. Edington[28] has recently made a very thorough study of another guinea pig epidemic caused by *B. aertrycke* in which this organism was recovered regularly from the blood, intestine, gall-bladder, liver, spleen and urine. Blood studies showed a leucopenia varying from 2,000 to 4,000 cells per cmm. with no striking change in the differential count. The lesions found were empyema of the gall-bladder, necrosis in the spleen and liver, hyperemia of the intestine and occasional small ulcerations in the lymphoid follicles of the mucosa, accumulations of phagocytes with necroses in the mesenteric lymph nodes, catarrhal inflammation of urinary bladder, purulent endometritis, infrequent pneumonia and cloudy swelling of kidneys. No mention was made of the bone marrow.

Though the literature contains several references,[29, 30, 31] other than instances of food poisoning, to human cases of fatal infection with *B. aertrycke*, none of them has resembled the disease caused by this organism which we have studied in pigeons. From the accounts of such cases it seems that *B. aertrycke* was recovered only from the blood, and was never proved to be the cause of any of the lesions present.

SUMMARY AND CONCLUSIONS

The apparently spontaneous development of a fatal disease in undernourished pigeons is reported which is characterized by anemia, marked myeloid hyperplasia of the bone marrow, striking increase of the myeloid elements of the blood, and extensive infiltration of the liver and kidneys with myeloid tissue. In addition to these myeloid changes, large, nodular, often necrotic masses of mononuclear phagocytic cells are frequently found scattered throughout the liver, spleen, kidneys and bone marrow.

A small, Gram-negative bacillus, regularly recovered in pure culture from the blood, liver, kidney, spleen, and bone marrow of these cases, has been identified as *B. aertrycke*. In sections, the bacteria are found to be present in the foci of mononuclear cells, but do not occur within the collections of myelocytes.

Disease has been produced experimentally in normal pigeons by the intraperitoneal injection of liver emulsion made from naturally infected birds, intraperitoneal injection of *B. aertrycke* derived from the same source, and also by oral administration of single large doses of broth cultures of this organism.

Bacteria-free filtrates of broth cultures of *B. aertrycke* have had no demonstrable effect upon normal pigeons when injected or administered orally in single large doses.

Attention is called to the frequency with which pathological changes occur in the tissues of apparently normal birds.

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DESCRIPTION OF PLATES

PLATE 67

Cells stained supravitally with neutral red and Janus green from the peripheral circulation of pigeons in this experimental series.

FIG. 1. Young adult eosinophilic leucocyte showing mitochondria and a mixture of spheres and rods making up the specific granules. These are the first changes to appear in the circulating granulocytes of the pigeon under stress. Pigeon 70, April 16, 1929, size 9 × 12 microns.

FIG. 2. Young adult eosinophilic leucocyte with mitochondria. Pigeon 65, peripheral blood, April 3, 1929, size 9 × 9½ microns.

FIG. 3. Atypical micro-eosinophil with swollen rods not infrequently found in the circulation as an indication of bone marrow stimulation. Pigeon 65, April 3, 1929, size 8 × 9 microns.

FIG. 4. Adult eosinophilic leucocyte showing the incomplete transition of spherical granules to rods and indicating a premature delivery of this cell to the circulation. Pigeon 65, April 3, 1929, size 10 × 15 microns.

FIG. 5. Eosinophilic myelocyte "B" with round nucleus, mitochondria and a moderate number of specific, spherical granules. Pigeon 65, April 3, 1919, size 10 × 10 microns. See erythrocyte for relative size.

FIG. 6. Eosinophilic myelocyte "A" with many mitochondria and few specific granules. Size 9 × 10 microns. Note the different stages of maturation found within the eosinophilic group on the same day from Pigeon 65, April 3, 1929.

FIG. 7. Eosinophilic myelocyte "C," showing mitochondria and spherical granules arranged about the centrosphere, a not infrequent arrangement in the myelocytic stage. The slightly indented achromatic nucleus contains one nucleolus. Pigeon 50, March 22, 1929, size 10½ × 12 microns.

FIG. 8. Myelocyte "B" contains approximately one-half the final concentration of specific granules found in the fully mature eosinophil and many mitochondria. The area about the centrosome is clear of granules. The nucleus shows very little chromatin structure at this stage of development. Pigeon 50, March 22, 1929, size 15 × 15 microns.

FIG. 9. This myelocyte "A" has relatively few specific granules, several vacuoles, and many mitochondria. The nucleus contains two nucleoli and very little chromatin. Size 12 × 13½ microns. Cells 7, 8 and 9 show the various stages of myelocytic maturation found on the same day in the blood of Pigeon 50, March 22, 1929.

FIG. 10. Myelocyte "B." Pigeon 65, April 4, 1929, size 9 × 12 microns.

FIG. 11. Myelocyte "A." Pigeon 65, April 4, 1929, size 10 × 14 microns.

FIG. 12. Monocyte from the peripheral blood of Pigeon 50, March 21, 1929. Note the slightly different shade of neutral red reaction in the vacuoles of this cell as contrasted with the granules in the myelocytes of Figs. 7, 8 and 9 from the same bird. See accompanying erythrocyte for relative size.

FIG. 13. Monocyte from Pigeon 65, April 4, 1929. Contrast this cell with the myelocytes of Figs. 10 and 11 taken from the same bird on the same date. Size 9 × 10½ microns.

FIG. 14. Small lymphocyte with many mitochondria and a few scattered neutral red vacuoles. Pigeon 67, April 4, 1929, size 9 × 9 microns. See accompanying erythrocyte for relative size.

PLATE 68

FIG. 15. Liver of Pigeon R714, naturally infected with *B. aertrycke*, showing unusually large collection of monocytic cells, in which there is a necrotic area containing Gram-negative bacilli. Hematoxylin-eosin stain. ×62.

FIG. 16. Smaller collection of monocytic cells in liver of Pigeon 24, experimentally infected with *B. aertrycke*. There are bacteria and a minute area of necrosis in the center about which is heavy infiltration with adult polymorphonuclear leucocytes. Hematoxylin-eosin stain. ×300.

FIG. 17. Low power picture of liver of Pigeon 14, experimentally infected with *B. aertrycke*, showing general distribution of infiltrations of myeloid cells. ×62.

FIG. 18. Small branch of portal vein with surrounding zone of myelocytes. Experimental infection *B. aertrycke*, Pigeon 14. Hematoxylin-eosin stain. ×300.

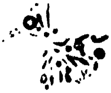
PLATE 69

FIG. 19. Myelocytes surrounding portal vein. A small portion of the vessel filled with erythrocytes may be seen in the upper left-hand corner. Natural infection with *B. aertrycke*, Pigeon 14. Hematoxylin-eosin stain. ×1300.

FIG. 20. Infiltration of kidney with myelocytes. Experimental infection with *B. aertrycke*, Pigeon 14. Hematoxylin-eosin stain. ×158.

FIG. 21. Bone marrow from central portion of shaft of radius of Pigeon 30 dying of experimental *B. aertrycke* infection. The partially collapsed capillaries are marked out by the characteristic nuclei of erythrocytes. All of the remaining cells are myelocytes of varying degrees of maturity. The more mature forms appear darker than the others due to their greater number of granules. The degree of myeloid hyperplasia may be appreciated by bearing in mind the fact that the capillary network of this portion of the marrow of normal pigeons contains only fat among its meshes. Hematoxylin-eosin stain. ×300.

FIG. 22. Oil-immersion picture from same marrow as Fig. 7, illustrating the pale staining, myeloid forms with only a few granules in their cytoplasm. A few mature myelocytes with their full quota of granules are also seen. Hematoxylin-eosin stain. ×1300.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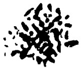
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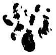
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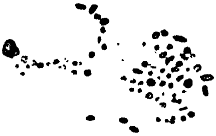
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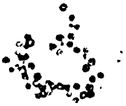
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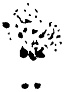
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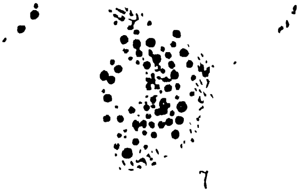
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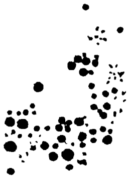
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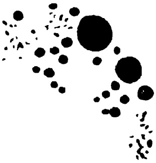
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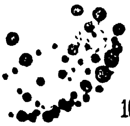
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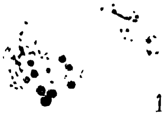
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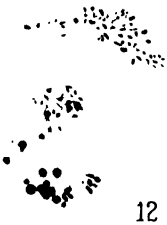
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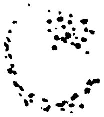
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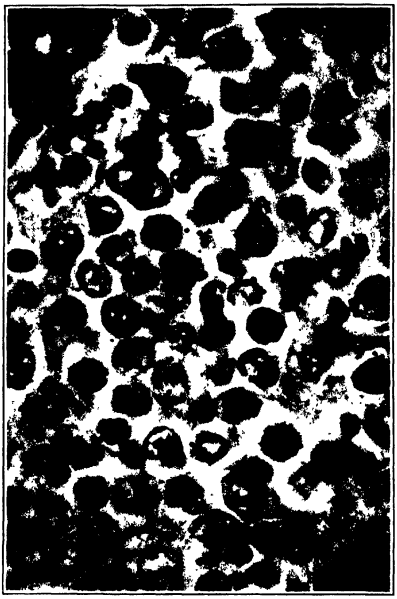

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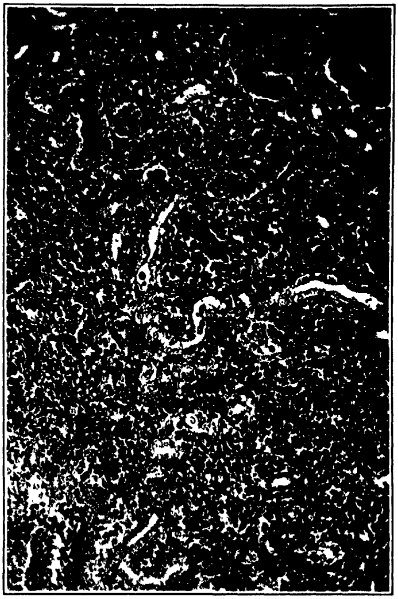

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ON THE SPECIFICITY OF SEROLOGICAL REACTIONS WITH SIMPLE CHEMICAL COMPOUNDS (INHIBITION REACTIONS)

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Whereas the usual serological reactions involve the use of high-molecular antigens of unknown constitution, a method has been described in a previous paper (1) which permits of the application of serological reactions to compounds of simple chemical composition. The method is based upon the possibility of partially synthesizing antigens (2, 3), by attaching to proteins chemical substances of simple structure as can be done by coupling with diazo compounds. Immune sera produced by injecting such "synthetic antigens" exhibit a specificity depending on the simple substances forming a part of the complex, particularly if the test antigen used contains a protein different from that used for immunization.

In ordinary precipitin tests, it has long been noticed that the reactions can be inhibited by addition of an excess of the antigen. Applying this observation to artificial complex antigens, it has been shown that the precipitin reactions of these substances are often inhibited specifically by the addition of a sufficient quantity of the same or a similar chemical substance to that used in building up the antigenic complex (1). These observations have since been confirmed by Klopstock and Selter (4), Avery and Goebel (5), and by ourselves.

The inhibition reactions with substances of known chemical structure can be used for the study of serological specificity in general, and in this regard they have certain advantages over the precipitin or complement fixation tests made with the full artificial antigens. Thus, in the first place it is not necessary to use substances which can be combined with proteins and, consequently, a greater variety of compounds can readily be subjected to the test. Furthermore an advantage may arise from the circumstance that the possible influence of the protein part of the antigen, and of the mode in which the specific part is attached, are entirely excluded.

A particular application of the inhibition test has been suggested previously (1), namely the determining of the nature of the specific group in an antigen in cases in which this is not known. This plan has been followed in our laboratory by Wormall (6), who was able to demonstrate that in iodized protein the reacting group is the diiodotyrosine radical, since the precipitin reaction of the protein can be inhibited specifically by diiodotyrosine but not by ortho-iodophenol or potassium iodide. An essentially similar method has repeatedly been employed in recent studies on the specificity of ferments.

In the present paper, some additional results are discussed which were obtained in the course of serological studies on azoproteins.

Influence on the Specificity of the Position of Substituents in Aromatic Compounds

The significance of the position of substituents in the benzene ring for the specificity of precipitin reactions was established in tests (2) with immune sera, reacting on antigens containing various aromatic acids, *e.g.*, *o*-, *m*-, and *p*-aminobenzoic acid antigens.*

Similar conditions appeared to prevail in the inhibition tests. In fact, by means of an immune serum for *p*-aminobenzoic acid it was possible to differentiate various ortho- and para-monosubstituted benzoic acids, regardless of the nature of the substituent which was variously OH, CH_3, Br, Cl, or NO_2 (1). For, in all instances, solutions of the sodium salts of the *p*-acids were much more active than those of the *o*-acids, while the *m*-substituted compounds were intermediate in activity.

In order to obtain further information it seemed necessary to examine also immune sera for *o*- and *m*-substituted antigens which had not been studied sufficiently in the previous experiments. The antigens used for immunization were prepared by coupling diazotized *o*-, *m*-, and *p*-aminobenzoic acids with horse serum. For the technique of the prep-

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* For the sake of brevity, the immune sera and antigens prepared with azoproteins will be designated by the compounds used for diazotization and subsequent coupling.

TABLE I

The following substances were used for the inhibition tests: (1) ortho-aminobenzoic acid; (2) meta-aminobenzoic acid; (3) para-aminobenzoic acid; (4) ortho-methylbenzoic acid; (5) meta-methylbenzoic acid; (6) para-methylbenzoic acid; (7) ortho-chlorobenzoic acid; (8) meta-chlorobenzoic acid; (9) para-chlorobenzoic acid; (10) ortho-bromobenzoic acid; (11) meta-bromobenzoic acid; (12) para-bromobenzoic acid; (13) ortho-oxybenzoic acid; (14) meta-oxybenzoic acid; (15) para-oxybenzoic acid; (16) ortho-nitrobenzoic acid; (17) meta-nitrobenzoic acid; (18) para-nitrobenzoic acid; (19) benzoic acid.

The tests with the meta-aminobenzoic acid immune serum No. 2 were made at 37°, the other tests at room temperature.

Immune serum for	Serum No.	Reading after	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	C
Ortho-aminobenzoic acid	1	15 min.	0	0	±	0	0	0	0	0	0	0	0	0	0	0	+̲	0	0	0	0	+
		1 hr.	0	tr.	+	0	0	f. tr.	0	0	0	0	0	0	0	t̲r̲.	+	0	0	tr.	0	+±
		2 hrs.	0	±	+	0	0	tr.	0	0	f. tr.	0	0	0	0	±	+	0	0	t̲r̲.	0	+±
		3 hrs.	0	±	+	0	0	±	0	0	f. tr.	0	0	0	0	+	+	0	0	±	0	+±
		Night in ice box	±	+	+±	±	±	+	t̲r̲.	t̲r̲.	+̲	t̲r̲.	t̲r̲.	±	t̲r̲.	+	+±	t̲r̲.	t̲r̲.	+	±	+±
Meta-aminobenzoic acid	2	15 min.	0	0	±	±	0	0	f. tr.	0	0	tr.	0	0	f. tr.	tr.	±	t̲r̲.	0	0	0	+±
		1 hr.	tr.	0	+	+	0	tr.	±	0	0	±	0	0	tr.	t̲r̲.	+±	+̲	0	f. tr.	0	++±
		2 hrs.	±	0	+	+	0	tr.	+̲	0	0	+̲	0	tr.	tr.	t̲r̲.	+±	+̲	0	tr.	0	+++
		Night in ice box	±	tr.	+±	+	f. tr.	±	+	0	±	+̲	f. tr.	+̲	±	+̲	+±	+̲	0	±	t̲r̲.	+++
Meta-aminobenzoic acid	3	15 min.	tr.	f. tr.	±	±	0	t̲r̲.	t̲r̲.	0	f. tr.	tr.	0	0	0	f. tr.	±	t̲r̲.	0	tr.	0	+±
		1 hr.	+̲	t̲r̲.	+	+	0	+̲	+̲	0	±	±	0	t̲r̲.	tr.	±	+	+̲	0	+̲	tr.	++
		2 hrs.	+̲	±	+	+	0	+	+	0	+̲	+	0	±	t̲r̲.	+̲	+	+	0	+	±	++
		Night in ice box	+	+	+	+	+̲	+	+	+̲	+	+	±	+	+	+	+±	+±	±	+±	+̲	++
Para-aminobenzoic acid	4	15 min.	tr.	t̲r̲.	0	±	0	0	t̲r̲.	0	0	±	0	0	f. tr.	±	0	±	0	0	f. tr.	+
		1 hr.	t̲r̲.	±	0	+̲	f. tr.	0	+̲	f. tr.	0	+	0	0	tr.	+̲	0	+	0	0	tr.	+±
		2 hrs.	±	+̲	0	+	t̲r̲.	f. tr.	+	tr.	0	+	f. tr.	0	t̲r̲.	+̲	0	+	f. tr.	0	tr.	+±
		3 hrs.	±	+̲	0	+	t̲r̲.	f. tr.	+	tr.	0	+	f. tr.	0	±	+̲	f. tr.	+	f. tr.	0	±	+±
		Night in ice box	±	+̲	tr.	+	±	t̲r̲.	+	±	tr.	+	t̲r̲.	tr.	±	+̲	tr.	+	t̲r̲.	tr.	±	++

aration of the antigens, and the immunization, we refer to previous communications (1, 7).

For the inhibition tests 1 millimol of each substance to be tested was dissolved in water by the aid of sodium hydroxide, and the solution made neutral to litmus and brought up to a volume of 10 cc. Unless otherwise stated, in each test 0.05 cc. of this solution was mixed with 0.2 cc. of the diluted antigen (made with chicken serum) previous to the addition of the immune serum. The control tube (C) contained only antigen and immune serum. The dilutions of the antigens are in terms of a 5 per cent stock solution. The intensity of the precipitin reaction is indicated as follows: 0, f. tr. (faint trace), tr. (trace), $\underline{\text{tr}}$. (strong trace), ±, $\underline{+}$, +, +±, etc.

The results in Table I are what would be expected from former findings, that is, comparing *o*- and *p*-immune sera and the *o*- and *p*-substances, the inhibition was always more intense with the "homologous" substance. Also the inhibition of the precipitation by the *m*-aminobenzoic immune sera was in general strongest with meta-substituted compounds, though in the case of the oxybenzoic acids the inhibition effect of the ortho-substance was somewhat greater than that of the meta-substituted acid. On comparing *o*- and *p*-derivatives of benzoic acid, it was seen moreover that substitution in the "heterologous" position often diminishes considerably the inhibition reaction as compared not only with the "homologous" substance, but also with the non-substituted benzoic acid.

An influence of greater or lesser strength of the acids seems to be excluded as the cause of these effects, because the regularities observed are of the same sort for substituted acids which differ widely in their strength, and also because of the results with the *m*-substituted substances. As suggested previously (1, 2), the most probable explanation is that the outcome of the reactions in the cases under consideration depends on the steric correspondence in the position of the substituents in the immunizing antigens and the compounds employed for inhibition, even though the substances examined differ in their composition from the specifically binding groupings in the antigens.

A relationship of the observations described to the phenomenon of ferment specificity is indicated by the findings of Waldschmidt-Leitz and Balls (8), who reported that of the isomer (chloroacetylamino) benzoic acids only the meta-compound is hydrolized by pancreatic carboxypolypeptidase, a fact which the authors also explain on the basis of steric configuration. Other similar instances are the inhibition of the action of tyrosinase by aromatic acids (9), and the oxidation of various substrates by this enzyme (10). It is noteworthy that a specificity depending on the position of aromatic substituents has been observed recently in the skin reactions of a patient sensitive to resorcinol (meta-dihydroxybenzene) by Nathan and Stern (11). This patient did not react to either ortho- or para-dihydroxybenzene, nor to the methyl ethers of resorcinol, nor to phenol or pyrogallol.

Not all sera are equally well suited for the demonstration of the inhibitory effects. Thus with some sera which fell off after long storage and also with some fresh sera, the inhibition by benzoic acid was weaker than usual and the regularities described were blurred. In some such instances it was possible to increase the inhibition by heating the immune sera for 1 hour at 55°C. or by setting up the tests at that temperature.

Inhibition Effects by Fatty and Cyclic Acids

In the tests described previously (1), the reaction of a serum for *p*-aminobenzoic acid antigen was found to be inhibited by a variety of aromatic acids, *i.e.* benzoic acid and substituted benzoic acids, but not distinctly by several aliphatic acids tested. Since benzoic acid caused practically no interference with the precipitin reaction by sera for *p*-arsanilic acid or *m*-aminobenzenesulfonic acid—which, in turn, are inhibited by aromatic arsinic or sulfonic acids—it is plain that the effect produced by benzoic acid depends on the constitution of the substance containing a carboxyl group bound to the benzene ring, a structure which corresponds to that of the binding group in the antigen. In recent experiments it was, however, found that aliphatic carbonic acids, especially the higher ones such as caproic and heptylic acids, also inhibit the reaction of immune sera for aminobenzoic acids.

As Table II shows, the effect increased with the length of the chain and was most marked with the caproic and heptylic acids. Acids with much longer chains could not be properly tested on account of the turbidity of the neutral solutions, but from a test with caprylic acid it would seem that the action may be stronger with the higher members of the series. Consequently it is possible that the phenomena, at least in part, are due to the physicochemical properties of high fatty

TABLE II

The following substances were used for the inhibition tests: (1) benzoic acid; (2) cyclohexane carboxylic acid; (3) acetic acid; (4) propionic acid; (5) normal butyric acid; (6) isobutyric acid; (7) normal valeric acid; (8) isovaleric acid; (9) methylethylacetic acid; (10) normal caproic acid; (11) isocaproic acid; (12) diethylacetic acid; (13) normal heptylic acid.

Immune serum for	Serum No.	Reading after	1	2	3	4	5	6	7	8	9	10	11	12	13	C
Ortho-aminobenzoic acid	5	15 min.	0	0	+±	+±	+	+	+̲	+̲	+̲	tr.	±	+	±	++
		1 hr.	0	+̲	++	++	+±	+±	+	+±	+±	+	+	+±	+	++±
Para-aminobenzoic acid	6	5 min.	0	0	+	+̲	±	+	±	+̲	+̲	tr.	±	+	f. tr.	+±
		1 hr.	0	+̲	++	++	+±	++	+±	+±	+±	+	+±	++	+	+++

TABLE III

The following substances were used for the inhibition tests: (1) benzoic acid; (2) α-thiophene carboxylic acid; (3) nicotinic acid; (4) cinchonic acid; (5) furan carboxylic acid; (6) picolinic acid; (7) cyclohexane carboxylic acid; (8) α-pyrrole carboxylic acid; (9) α-naphtoic acid; (10) β-naphtoic acid; (11) acetic acid; (12) normal caproic acid; (13) normal caprylic acid.

Immune serum for	Serum No.	Reading for	1	2	3	4	5	6	7	8	9	10	11	12	13	C
Ortho-aminobenzoic acid	1	15 min.	0	0	tr.	0	tr.	±	f. tr.	tr.	0	0	+	tr.	f. tr.	+
		1 hr.	0	0	±	0	±	+̲	tr.	+̲	0	0	+	±	tr.	+
		2 hrs.	0	f. tr.	+	f. tr.	+	+	±	+	0	f. tr.	+±	+	±	+±
Meta-aminobenzoic acid	2	15 min.	0	f. tr.	tr.	tr.	t̲r̲.	±	±	±	0	0	+	±	±	+
		1 hr.	f. tr.	tr.	t̲r̲.	t̲r̲.	±	+	+̲	+	0	0	+±	+	+̲	+±
		2 hrs.	t̲r̲.	±	±	+	+̲	+±	+±	+±	0	0	++	+±	+±	++
Meta-aminobenzoic acid	3	15 min.	0	f. tr.	t̲r̲.	±	±	±	±	±	0	f. tr.	+±	+	±	+±
		1 hr.	tr.	tr.	±	+	+	+	+	+	f. tr.	tr.	++	+	+	++
		2 hrs.	±	±	+	+±	+±	+±	+±	+±	tr.	±	++±	+±	+±	++±
Para-aminobenzoic acid	4	15 min.	f. tr.	0	tr.	f. tr.	t̲r̲.	±	t̲r̲.	±	f. tr.	0	+	±	±	+
		1 hr.	tr.	f. tr.	±	t̲r̲.	±	±	±	+̲	tr.	0	+	+̲	+̲	+
		2 hrs.	t̲r̲.	tr.	+̲	±	+̲	+	+	+	±	0	+±	+	+	+±

acids. In this connection it should be mentioned that salts of cholic and desoxycholic acids appear to inhibit precipitin reactions in general. On the other hand, the chemical structure, particularly the presence of the carboxyl group, must play a part, since other precipitin reactions, *e.g.* that of an immune serum for arsanilic acid, are only slightly influenced by caproic or heptylic (or benzoic) acids. The fact that these acids have very little effect on precipitin reactions of proteins or carbohydrates, such as horse serum or a precipitable substance of pneumococci, may be attributable to the more intricate constitution of the specifically reacting groups in these antigens.

Table III illustrates the inhibitory action of some cyclic acids on the precipitation by *o*- and *p*-aminobenzoic acid immune sera.

The tests show that not only benzoic acid but also other cyclic acids exert inhibiting effects. Intense inhibition was caused by α-thiophene carboxylic acid and α- and β-naphtoic acids, the result with the former being in good agreement with the close chemical similarity between α-thiophene carboxylic acid and benzoic acid.

Inhibition Reactions of Pronounced Specificity

The experiments reported in the present and a previous communication show that the reactions of certain immune sera can be inhibited by numerous substances, provided these are chemically somewhat similar to the reacting group of the antigen used. Such is the case when the groupings responsible for the specific precipitation have a simple structure, as for example in the antigens prepared with aminobenzoic acids. Extending the well known simile of E. Fischer, these instances may be compared to the unlocking of a simple lock by various keys or of various locks by a simple key. Accordingly it appears that the inhibitory effect can result from weak affinities which would not be sufficient for causing specific precipitation, *e.g.*, a serum for para-aminobenzoic acid would be inhibited to a certain degree by ortho-aminobenzoic acid but would not precipitate test antigens built up from the latter substance.

It has already been suggested that the range of reactivity is narrowed in cases in which either the antigen or the inhibiting substance is of somewhat more complex chemical structure. Inhibition reactions with azodyes (1) are instances of the sort. Since this view has not been checked in any case by tests with numerous compounds of widely varied composition, and on account of the results reported in the preceding section, it seemed desirable to verify it by experiments carried out on a larger scale. To this end, two antigens and the corresponding immune sera, studied previously (12, 13), were selected with specific groups of more complicated constitution than those used in our first studies, namely two azoproteins, prepared from *l*-phenyl (para-aminobenzoylamino) acetic acid, and *l*-para-aminotartranilic acid. For the inhibition tests a large number of organic acids of widely varying chemical constitution were taken.

The following substances were used for the inhibition tests: (1) acetic acid; (2) normal butyric acid; (3) normal valeric acid; (4) isovaleric acid; (5) normal caproic acid; (6) isocaproic acid; (7) diethylacetic acid; (8) normal heptylic acid; (9) chloroacetic acid; (10) bromoacetic acid; (11) cyanacetic acid; (12) trichloroacetic acid; (13) β-iodopropionic acid; (14) lactic acid; (15) levulinic acid; (16) aminoisovaleric acid; (17) acrylic acid; (18) α-crotonic acid; (19) *d, l*-bromosuccinic acid; (20) citric acid; (21) fumaric acid; (22) glycocoll; (23) *d, l*-alanin; (24) phenylglycine; (25) *d, l*-phenylalanin; (26) *d, l*-leucine; (27) histidine; (28) benzoylglycine; (29) *d, l*-benzoylalanine; (30) *l*-asparaginic acid; (31) *l*-glutaminic acid; (32) camphoric acid; (33) cyclohexane carboxylic acid; (34) benzoic acid; (35) ortho-aminobenzoic acid; (36) meta-aminobenzoic acid; (37) para-aminobenzoic acid; (38) 4-chloro-3-aminobenzoic acid; (39) ortho-chlorobenzoic acid; (40) meta-chlorobenzoic acid; (41) para-chlorobenzoic acid; (42) ortho-bromobenzoic acid; (43) meta-bromobenzoic acid; (44) para-bromobenzoic acid; (45) ortho-nitrobenzoic acid; (46) meta-nitrobenzoic acid; (47) para-nitrobenzoic acid; (48) ortho-oxybenzoic acid; (49) meta-oxybenzoic acid; (50) para-oxybenzoic acid; (51) ortho-methylbenzoic acid; (52) meta-methylbenzoic acid; (53) para-methylbenzoic acid; (54) 2, 4, dinitrobenzoic acid; (55) 2, 4, 6, trinitrobenzoic acid; (56) anisic acid; (57) 1, 2, 5, dioxybenzoic acid; (58) 1, 2, 4, dioxybenzoic acid; (59) benzolsulfonic acid; (60) ortho-aminobenzolsulfonic acid; (61) meta-aminobenzolsulfonic acid; (62) para-aminobenzolsulfonic acid; (63) sulfosalicylic acid; (64) para-toluidinsulfonic acid; (65) para-azoxybenzoic acid; (66) phenylacetic acid; (67) mandelic acid; (68) benzilic acid; (69) vanillic acid; (70) cinnamic acid; (71) hydrocinnamic acid; (72) ortho-aminocinnamic acid; (73) meta-aminocinnamic acid; (74) para-aminocinnamic acid; (75) ortho-nitrocinnamic acid; (76) meta-nitrocinnamic acid; (77) para-nitrocinnamic acid; (78) ortho-cumaric acid; (79) phthalic acid; (80) isophthalic acid; (81) terephthalic acid; (82) α-thiophene carboxylic acid; (83) furan carboxylic acid; (84) meconic acid; (85) α-naphtoic acid; (86) β-naphtoic acid; (87) α-oxynaphtoic acid; (88) β-oxynaphtoic acid; (89) naphtionic acid; (90) α-pyrrole carboxylic acid; (91) picolinic acid; (92) nicotinic acid; (93) cinchonic acid.

For the tests 0.05 cc. of a neutral solution of the substance containing 0.5 millimol in 10 cc. water were used.

TABLE IV

Substance No.	Immune serum for levo-phenyl-(para-amino-benzoylamino) acetic acid		Immune serum for levo-para-amino-tartranilic acid		Substance No.	Immune serum for levo-phenyl (para-amino-benzoylamino) acetic acid		Immune serum for levo-para-amino-tartranilic acid	
1	+	++	+±	++±	48	+	++	+±	++
2	+	++	+±	++±	49	+	++	+±	++±
3	+	++	+±	++	50	+	++	+±	++
4	+	++	+±	++±	51	+	++	+±	++
5	+	++	+±	++±	52	+	++	+±	++
6	+	++	+±	++±	53	+	++	+±	++±
7	+	++	+±	++	54	±	++	+±	++
8	+	++	+±	++±	55	±	+±	+±	++±
9	+	++	+±	++	56	+	++	+±	++
10	+	++	+±	++	57	+	++	+±	++
11	+	++	+±	++	58	+	++	+±	++
12	+	++	+±	++	59	+	++	+±	++±
13	+	++	+±	++±	60	+	++	+±	++
14	+	++	+±	++	61	+	++	+±	++
15	+	++	+±	++	62	+	++	+±	++
16	+	++	+±	++	63	+	++	+±	++±
17	+	++	+±	++±	64	+	++	+±	++
18	+	++	+±	++	65	+	++	+±	++
19	+	++	+±	++	66	±	++	+±	++
20	+	++	+	++	67	±	++	+	++
21	+	++	+±	++	68	±	++	+±	++
22	+	++	+±	++	69	+	++	+±	++±
23	+	++	+±	++	70	±	++	+±	++
24	±	++	+±	++	71	±	++	+±	++
25	+	++	+±	++	72	+	++	+±	++±
26	+	++	+±	++	73	+	++	+±	++±
27	+	++	+±	++	74	+	++	+±	++
28	±	++	+±	++	75	tr.	+	+±	++±
29	±	++	+±	++±	76	±	++	+±	++±
30	+	++	+±	++±	77	±	++	+±	++
31	+	++	+±	++±	78	+	++	+±	++
32	+	++	+±	++	79	+	++	+±	++±
33	±	++	+±	++±	80	+	++	+±	++±
34	+	++	+±	++±	81	+	++	+±	++
35	+	++	+±	++	82	±	++	+±	++±
36	+	++	+±	++	83	+	++	+±	++±
37	+	++	+±	++	84	+	++	+±	++±
38	+	++	+±	++	85	±	++	+±	++
39	+	++	+±	++±	86	±	+±	+±	++
40	+	++	+±	++	87	+	++	+±	++
41	+	++	+±	++±	88	+	++	+±	++
42	+	++	+±	++	89	+	++	+±	++
43	±	+±	+±	++±	90	+	++	+±	++
44	+	++	+±	++±	91	+	++	+	++
45	+	++	+±	++±	92	±	++	+±	++
46	+	++	+±	++	93	±	+±	+±	++
47	+	++	+±	++	Hom. subst.	0	±	0	0
					Control	+	++	+±	++

In the table, the first column gives the readings after 2 hours at room temperature for each of the two immune sera, the second column those after standing overnight in the ice box "Hom. subst." stands for "homologous substance," namely: levo-phenyl (para-aminobenzoylamino) acetic acid, or levo-para-aminotartranilic acid.

It is seen from Table IV that in the tests with the first immune serum there occurred some inhibitions with non-homologous substances which, with one exception, disappeared or became very weak on longer standing. The homologous substance gave the strongest effect. With the immune serum for aminotartranilic acid only three heterologous substances showed relatively weak inhibition in the first reading, whereas the later reading demonstrated a perfectly specific reaction of the homologous substance. The inhibition with the homologous substance was complete also with one-half of the quantity used in the experiment presented and distinct with one-eighth of that quantity.

It may be concluded, therefore, that under appropriate conditions the inhibition tests exhibit a high degree of specificity, comparable to that of the common serological phenomena. Thus, the peculiar specificity displayed in serum reactions is not limited to high molecular colloids, but can be demonstrated as well with relatively simple crystallized substances, and is largely independent of the molecular size.

SUMMARY

Experiments are described which confirm the result that the specificity of inhibitory reactions involving substituted aromatic acids is decidedly influenced by the position of the substituent.

When antigens with specific groups of very simple constitution are used for the tests, inhibiting effects are obtained also with substances distantly related to those determining the reactivity of the antigens. On the other hand, if antigens are built up from protein and chemical compounds of somewhat higher complexity, the specificity of the inhibition reactions with synthetic crystallized substances is of the same order as that of the usual serum reactions; in other words, it is possible to distinguish such compounds by serological tests as readily as proteins can be differentiated with the aid of precipitating sera.

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THE INDUCED DEVELOPMENT AND HISTOGENESIS OF PLASMA CELLS

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PLATES *30* AND *31*

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It is known that plasma cells occur normally in the connective tissues of warm blooded animals and that they are increased in numbers around various pathological lesions. In a study of the cellular reactions to the different chemical fractions of the tubercle bacillus, Sabin, Doan, and Forkner (1) reported the presence of these cells in rather large numbers in the omenta of rabbits after intraperitoneal injections of tuberculo-protein.

The present work was undertaken to determine the effectiveness of the tuberculo-protein as a stimulant for plasma cells; and to use the resulting proliferations of plasma cells for a study of their histogenesis.

If the plasma cell is accepted as a definite cellular entity, it must have a maturation cycle, as have the myeloid elements and the lymphocyte (2). The cellular reaction following intraperitoneal injections of tuberculo-protein has made it possible to follow the evolution of plasma cells from primitive undifferentiated mesenchymal cells, through maturity, into Russell body cells and other degenerative forms.

The name plasma cell was introduced by Waldeyer (3) in 1875. From his work it is not clear that he described a specific type of cell. A definite cell strain was first recognized by Cajal (1890 (4)) in the condylomata of syphilis. He named them *cells cyanophils*. Unna (1891 (5)) found cells in the lesions of dermal tuberculosis which he called plasma cells. He described them as having a basophilic, spongy, or granular cytoplasm, the granuloplasm. Their nuclei were either centrally or eccentrically placed, and the chromatin content and arrangement were variable. His only constant specializing criterion was basophilic granuloplasm, and as a result of this he included under the category of plasma cells many cells with basophilic cytoplasm, which were probably derivatives of other strains.

Marshalkó (1895 (6)) selected certain morphological characteristics as the essential criteria of the typical plasma cell. By his definition such a cell must have basophilic cytoplasm, showing a clear area in the center, be round or oval, have a round, eccentrically placed nucleus with the chromatin condensed near the nuclear membrane and arranged radially in blocks. Cells that have these criteria are known today as typical plasma cells. It was Marshalkó's belief that they originated from hematogenous lymphocytes.

Downey's (7) discussion of the controversy over the origin of plasma cells shows that many believe them to be derived from lymphocytes, either hematogenous or histogenous, while others think they take origin from primitive mesenchymal cells. He gives evidence to support the view that they are derived from both types of lymphocytes, but in greater numbers from the hematogenous variety. In describing plasma cells in the mesentery of the frog, he shows that the Russell body cells are the end stage of their development and are present normally. The Russell body cells are plasma cells which have fulfilled a cellular secretory function, having built up some material in their cytoplasm, which first appears as granuloplasm, and later, becoming hyalinized, is present as the Russell bodies. An excellent review of the literature has been given recently by Michels (8).

Although plasma cells are found in normal connective tissues, they are present in the greatest number in organs which act as bacterial filters or which presumably have a detoxifying function, notably the lymph nodes and omentum. This, together with their presence in certain pathological lesions, suggests that they act to detoxify bacterial and other toxins.

Methods

Three tuberculo-proteins were used to stimulate the production of plasma cells, as follows: (*a*) one isolated by Johnson and Coghill (9), designated water-soluble 304; (*b*) an alkali-soluble fraction, also isolated by Coghill (10); and (*c*) a water-soluble material similar to that of Johnson but prepared by the Mulford Company (MA-100). The first two, because they were slightly acid, were suspended in distilled water or salt solution and caused to dissolve by the addition of enough 1 per cent NaOH to give a neutral reaction to litmus. The Mulford protein, MA-100, was received in salt solution and injected in this form.

Eighteen animals were employed in the experiments. To stimulate the cells in the omentum, seventeen of them were given intraperitoneal injections of one of the proteins. Two of the seventeen animals were also given intravenous injections, and three were injected subcutaneously. The remaining animal was given intravenous injections only.

Table I indicates the number of injections, the type and amount of protein each animal received, and the interval between the last injection and autopsy.

After its course of injections, each animal was killed by intravenous injection of air and an autopsy was performed. The omentum was spread over a slide prepared with neutral red and Janus green for study by the supravital method. Lymph nodes were scraped and prepared for supravital study. Parts of the various organs which seemed to be injured by the action of the protein were fixed in Helly's solution and then they were stained with hematoxylin and eosin, or with Giemsa, for microscopic study.

TABLE I

Rabbit No.	Type of protein	Number of injections	Amount of each injection	Interval between last injection and autopsy
			mg.	*days*
R 1536	304	3	20	7
R 1166	304	3	20	10
R 1734	Alkali-soluble	5	10	7
R 1803	Ma-100	5	20	11
R 1600	304	4	20, ip. 10, iv.	2
R 1105	304	6	20	2
R 1735	Alkali-soluble	10	10	6
R 1151	304	12	10	2
R 1153	Alkali-soluble	12	10	2
R 1112	304	7	14, ip. 6, sc.	5
R 1109	304	10	20	1
R 1104	304	12	20	3
R 1804	MA-100	13	20	21
R 1106	304	15	18, ip. 6, sc.	4
R 1102	304	20	20	7
R 1168	Alkali-soluble	25	10, ip. 5, sc. (5 injections)	2
R 1608	304	36	10, iv.	2
		10	10, ip.	2
R 1211	304	48	10, iv.	Not dead

Induced Foci of Plasma Cells

At autopsy the omentum usually showed a hyperplastic reaction; it was thickened, had an increase of milk spots, as well as much new fibrous tissue and many new blood vessels. In several of the animals (R 1102, R 1168, R 1153, R 1735[1]) small white nodules were found attached to the omentum, body wall, and large intestine. Often there was inflammation of the body wall, with a new growth of small vessels and a roughening of the whole surface.

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[1] These are serial numbers of the work of the department covering a term of years.

The omenta of five normal rabbits (R 650, R 651, R 652, R 578, and R 879) were studied in comparison with those of the experimental animals. Plasma cells were found in only two of these and in rather small numbers. The greater part of each normal omentum, as seen in section, was made up of fat cells. The remainder of the cellular structure was made up of undifferentiated cells, small groups of lymphocytes, a few monocytes, and a network of mesothelial cells.

Table II indicates the organs in which plasma cells were found in the experimental animals. The omentum was the site of the greatest proliferation of these cells, although they were present in the liver, *spleen, cecum, body wall, and scattered lymph nodes. Two animals which received intravenous injections also showed a few plasma cells in the bone marrow. In the omentum the plasma cells were found in* the milk spots, about the blood vessels, and scattered through the connective tissue. Some omenta were so stimulated by the protein that the plasma cell was the predominating cell element (Fig. 5). Where they were so vastly increased in numbers, the lymphocytes were also increased. New lymph follicles with germinal centers could be made out where the irritation had been greatest. Plasma cells were not seen in these lymph follicles, although they often encroached on the borders of them. On the whole the two strains of cells were quite definitely segregated, but one animal (R 1168) had areas in the omentum in which plasma cells and lymphocytes were mixed. The lymphocytes, as a rule, lay in the spaces between the fat cells, and the plasma cells always in the diffuse connective tissue.

The white nodules noted on the omentum, body wall, and large intestines were made up of necrotic material. Many of the cellular elements were still recognizable in them as plasma cells and leucocytes. The tissue surrounding these nodules was usually made up of connective tissue cells, including many plasma cells. One such nodule was taken from the large intestine of R 1735; another from the large intestine of R 1153. In these could be seen three stages of the growth of new fibrous tissue. There were areas of degenerating plasma cells and leucocytes, areas of deposition of fibrin and slight hemorrhage,

TABLE II

Organs in Which Plasma Cells Were Found

Rabbit No.	Omentum	Kidney	Liver	Spleen	Body wall	Small intestines Peyer's patches	Wall of large intestines	Bone marrow	Lung	Lymph nodes							Cecum
										Axillary	Submaxillary	Popliteal	Inguinal	Retroperitoneal	Tracheal	Mesenteric	
R 1112	++++	0	0	0		0		0				0	0			0	
R 1153	++++	0	+ Para-sites	0	++++		++++	0	0	0		0		0		0	
R 1105	++++	0	0	0				0		0	++++		0			0	
R 1151	++++	0	0	0	+++			0		0					++		
R 1106	++++		0	+				0				0	+				
R 1104	++++					0		0				0					
R 1109	++++	0	0	0				0	0	+		+	0			+	
R 1536	++++	0		0		0	0	0	++++	0	+					+	
R 1102	++++							0							+	+	
R 1600	+++	0	0	0					+	0			0			0	
R 1734	++++	0	+	0				0			+		0			0	
R 1168	++++	0	0	+	++++		++++	0				++ L 0 R	0			+	
R 1735	++++	0	0	0		++		0		+	+		0	0	0	0	0
R 1166	+++							0		0		0				++	
R 1608	++++	0	0	0				+	0		+++	0			0	+	
R 1803	++++	0	0	0		+		0	0	0	+	0	0			0	
R 1804	++	0	+++	0		0		0	0	0		0	+	0		0	

and areas of new fibrous tissue. About the base of the gland cells were many lymphocytes and many young plasma cells. One nodule from the body wall of R 1168 showed an infiltration of the plasma cells into the connective tissue between the muscle bundles. The lung of R 1536 contained an area of bronchopneumonia about which was a small amount of fibrous tissue. In this fibrous tissue and infiltrating into the pneumonic area were many plasma cells. There were also present small nodules of lymphocytes with germinal centers.

The animals which received the largest amounts of the protein over the longest periods of time (R 1102, R 1109, and R 1168) showed the greatest proliferation of plasma cells. These animals also revealed the greatest variety of stages in the life cycle of the plasma cell. The period elapsing between the last injection and the autopsy was a determining factor in the differentiation of the plasma cell. More true Russell body cells were found in the tissues of those animals, especially R 1804, in which this period was the longest. The largest numbers of degenerating plasma cells were not necessarily present in these animals, but time was probably not so much responsible for them as was crowding.

Histogenesis of Plasma Cells

Most of the plasma cells in both the stimulated tissues and the normal organs were of the typical Marshalkó variety. However, other plasma cells with definite characteristics of immaturity were always seen in or near all the groups of typical forms. There were also older degenerating cells, but these were rare except in the necrotic white nodules which were composed of them almost wholly.

The primitive mesenchymal cells and the young cells in the milk spots were the precursors of the plasma cells. Undifferentiated mesenchyme cells are normally present scattered diffusely in the connective tissues and lying along the blood vessels. The milk spots of the omentum are made up chiefly of relatively undifferentiated cells which have the potentiality of developing into monocytes or macrophages. These cells have been termed polyblasts by Maximow (11). Sabin *et al.* (1) have shown them to be transformed readily into monocytes after intraperitoneal injections of the tuberculo-phosphatide. Monocytes, lymphocytes, and cells which are more immature than these so called polyblasts are also found in the milk spots. These young cells in the milk spots also are often called primitive cells. In stained sections they appear slightly younger than blast forms of lymphocytes and leucocytes.

Two primitive mesenchymal cells are shown in Fig. 1 (see arrows). These cells were characterized by large nuclei and little if any cytoplasm. The structure of the nuclei was a fine network with little chromatin. This stained light gray-blue and the nucleoli, which were small and usually two or three in number, stained red or purple. When the cytoplasm was discernible, it was of a slightly basophilic quality. The development of the primitive cells into the Marshalkó type plasma cells was a graded transitional process in which both the nuclei and cytoplasm changed in staining quality and structure. The fact that the young cells were of different ages could be readily made out. Those slightly older than the primitive cells showed slight condensations of chromatin near the nuclear membranes. The nucleoli of these cells were somewhat larger than those in the undifferentiated cells, and the cytoplasm showed beginning basophilia. The grade of basophilia of the cytoplasm and the amount of condensation of chromatin of the nuclei were indices of how far advanced from the primitive cell any one of these had become. Many of this type of cell are shown in Fig. 1. The photograph was taken from a section of body wall of R 1168. A portion of the section was composed almost entirely of young cells and there were also a few lymphocytes. The part shown was from a nodule in the body wall which was almost tumor-like in its cellular picture. Typical plasma cells were seen in groups and scattered throughout all parts of the tissue surrounding the nodule.

As the young forms matured, they fulfilled the criteria of the Marshalkó plasma cells. Young cells were seen which had one or all of the specified characters. Some had a distinctly basophilic cytoplasm with a clear area in its center. The chromatin in the nucleus was so condensed as to give the appearance of the cart-wheel nucleus, a *Radkern*, and in most of this type of cell the nucleoli were retained. The nuclei were either eccentric or central in position.

In groups of the young plasma cells some were found with mitotic figures; one such is shown in Fig. 1. Reproduction of the plasma cell in this early phase by mitosis, though not rare, was not very common.

As the cells matured, the nuclei became smaller and the chromatin more condensed, especially at the nuclear membrane. The condensation of the chromatin was in blocks which gave the characteristic cart-wheel nucleus. The basophilic quality of the cytoplasm became more pronounced. Most of this type of cell had eccentrically placed nuclei and clear areas in the centers of their cytoplasm. In these mature cells the nucleoli were lost. These cells are shown in Fig. 2. In this figure are seen typical plasma cells and also two or three cells which have one or more of the criteria of Marshalkó plasma cells, but which are too young to have them all. There are also areas such as that represented in Fig. 5, in which all the cells are of the mature type. The plasma cells proliferated by both mitosis and amitosis, and this latter fact is shown by the presence of two or three nuclei, or by large cells budding smaller ones.

Even in the mature plasma cells, gradations of morphological character were evident. Not only were some obviously younger than the typical Marshalkó variety, but there were also older ones. The cytoplasm of these latter had become spongy and somewhat granular. The spongy cytoplasm conformed to the description of granuloplasm by Unna. The nuclei of cells with granuloplasm were usually smaller than those of the less mature cells. The cytoplasm of the younger cells, whether of the pre-Marshalkó type or of the Marshalkó type, appeared homogeneous in contradistinction to the granuloplasm.

Following the development of granuloplasm, the plasma cells began to degenerate. Two types of degeneration occurred. Some cells, as they became senile, lost their basophilic appearance, the nuclei became pycnotic, and often vacuoles appeared in the cytoplasm. There developed in certain of these cells hyaline bodies with the same appearance and staining reactions as the true Russell body cells. Most of the cells undergoing degeneration of this first sort were packed closely together, and it is possible that crowding led to their degeneration. The other type of degeneration was into characteristic Russell body cells. Some of the plasma cells developed acidophilic granules and hyaline or crystalline bodies appeared later in the cytoplasm; each of these bodies seemed to be a segregated part of the latter. The nuclei were fragmented or pycnotic and usually were in the eccentric position. The true Russell body cells were found infrequently in any of the areas which had been stimulated with the protein. The first type of degeneration was frequent wherever the plasma cells predominated in the tissues, as in the white nodules described above. True Russell body cells could be made out scattered through the matrix of aggregates of the sort. Fig. 3 shows degenerating plasma cells. A few of these contain acidophilic granules and hyaline bodies (Arrows A).

Supravital Studies

Supravital studies of plasma cells have been reported by Bloom (12), Forkner (13), Jackson *et al.* (14). Their descriptions are quite in agreement, except for the fact that Jackson did not find neutral red bodies in the cytoplasm constantly.

Omenta from normal rabbits and from rabbits injected with tuberculo-protein were examined with the supravital method. The stimulated omenta were studied first and the findings correlated with those of the fixed tissues. All omental spreads were fixed with methyl alcohol and stained with methylene blue and Giemsa after they had been studied by the supravital technique.

The plasma cells appeared round or oval, when stained supravitally, or they took on peculiar configurations when pressed by other cells. The cytoplasm was a yellowish gray and, in the spreads, the mitochondria were not seen unless a small amount of a 1:10,000 solution of Janus green was added. Mitochondria did not always stand out even after this treatment. When demonstrable, they were either scattered throughout the cytoplasm, or were grouped in the center of it. Neutral red bodies were rare. Occasionally, in very large cells, a thinning in the center of the cytoplasm represented the clear area so characteristic in fixed staining. The mitochondria were usually numerous, some cells having as many as 30 or 40 of them. They varied in size and in shape and were usually smaller than those in young lymphocytes. They took the forms of small rods or round dots, though the rod-shaped mitochondria were more frequently seen.

The nuclei of these cells were usually about the same shade of gray as the cytoplasm. This made them difficult to see, though the heaping up of the chromatin was often distinctive. If the cells were young enough, nucleoli stood out as small pearls. Differentiation of young cells from older ones was difficult to make out, because the characteristics of age were not marked in preparations made by this technique, except in the case of the Russell body cell.

Russell body cells were found in the tissues of three of the experimental animals. The cytoplasm of these cells appeared as gray as the cytoplasm of the younger cells. Some of the Russell body cells had from three to five cytoplasmic lobulations; others had lobulations which entirely filled the cells. Each lobulation seemed to be a segregated part of the protoplasm, and was smooth and non-granular. The nuclei were always eccentric in position and usually smaller than those of the less mature cells. The cells with the larger number of lobulations had no mitochondria, and the nuclei were so small that they could not be seen or else possibly were directed away from the microscope.

The cells described here as plasma cells were found singly and in groups throughout all stimulated omenta and were usually beneath the surface, so that they were difficult to bring into focus. They were most numerous along the blood vessels and in the milk spots. Lymphocytes were also seen, but as already mentioned, they were rarely in the groups of plasma cells.

The two animals which received intravenous injections of the protein developed as many as 4 per cent plasma cells in the peripheral blood stream on several occasions. Here they had the same tinctorial properties as in the omental spreads, but they usually contained from five to eight neutral red bodies grouped in the center of the cytoplasm. Maximow (11) has previously shown that the clear area is the site of the centrosome, and it is not therefore peculiar that there was a grouping of the vacuoles stained with neutral red around this space. The mitochondria stained more brightly and were seen with less difficulty than in the omental spreads. They were usually scattered throughout the cytoplasm. The nucleus often was very indistinct, being of about the same shade of gray as the cytoplasm.

Unstimulated omenta also contained cells which were recognized as plasma cells. In these omenta they were found rarely, although one animal (R 1606) had many of them.

By the supravital technique, plasma cells were differentiated from lymphocytes by their darker gray cytoplasm, eccentric nuclei, and the variations in size, staining quality, and distribution of mitochondria.

In the young lymphocytes the nuclei were usually large and the mitochondria stained with ease and were grouped about the periphery of the nucleus. The nucleus of the young lymphocyte stood out plainly, while that of the plasma cell was usually indistinct.

The plasma cells in these same omental spreads, when stained with methylene blue and Giemsa, were for the most part of the Marshalkó type. The cytoplasm of many of them had a tendency to be granular. Russell body cells had the same appearance as in tissues stained with hematoxylin and eosin. The nuclei of the typical plasma cell showed an unaltered chromatin arrangement, but stained light pink. As by the supravital technique, these cells were grouped about blood vessels, in the milk spots and scattered throughout the connective tissue stroma. In some parts of each omentum many lymphocytes were found, but only rarely amongst the plasma cells.

Some omenta showed increases in the number of fibroblasts and clasmatocytes. In these, often, the fibroblasts were seen with their fibrils about single plasma cells. This peculiar relationship is shown in Fig. 4.

Lack of Relationship of Plasma Cells and Lymphocytes

In many aspects plasma cells and lymphocytes resemble each other. Plasma cells are found where lymphocytes develop, and the stimulating effect of the tuberculo-protein causes many young lymphocytes and new lymph follicles, as well as plasma cells, to be formed. The plasma cells are found almost always in the connective tissues; even when found in the lymph nodes they are in the connective tissue cords. They are a constant element in the submaxillary lymph nodes and occasionally may be present in any other of the lymphoid structures (Table II). In the submaxillary nodes they are usually present in rather large numbers, while in other nodes they are few in number.

As a rule, plasma cells are absent from the popliteal and inguinal nodes. Because of this, and the fact that lymphocytes have been termed the precursors of plasma cells, these nodes were subjected to direct stimulation or irritation by the protein. To effect this stimulation of the inguinal nodes, two animals were injected subcutaneously in the groin. Another animal was given injections of the protein into its left foot pad so that the material would drain through the popliteal node.

R 1106 received fifteen injections of the material in the right groin, each of 6 mg. in 2 cc. of fluid. R 1112 received five similar injections in the right groin. R 1168 was given five injections of 5 mg. each in its left foot pad. When these animals were autopsied, supravital studies of the regional lymph nodes showed no plasma cells. The inguinal nodes of R 1106 were very much enlarged and contained epithelioid cells, but those of the other animals were not enlarged and were free from abnormal components.

The fixed sections of the right inguinal nodes of R 1106 contained few plasma cells, and there were a few scattered plasma cells in the left popliteal nodes of R 1168. The other regional nodes or those which were subjected to the irritation of the protein were free from plasma cells.

Young plasma cells differ much from young lymphocytes in their morphology. The nuclei of young plasma cells are denser in chromatin; they simulate the nuclei of the older cells, but are larger and usually contain nucleoli. The primitive cells, of course, are so undifferentiated that they have no specializing criteria. Primitive cells which form plasma cells are much like those that form lymphocytes. The cytoplasm of the youngest differentiated cells of the plasma cell series is usually basophilic, although not so markedly as that of older cells. Young lymphocytes appear much like the young plasma cells, although their nuclei are centrally placed, and often bean-shaped. The chromatin in the nuclei of the young cells of the lymphocyte series is usually in thread-like formations, not condensed near the nuclear membranes. The cytoplasm of these cells is slightly less basophilic than that of the plasma cells.. Mitotic figures are more frequently seen in groups of young lymphocytes than in plasma cells.

Young lymphocytes are best seen in or near the germinal centers of the lymph nodes. The mesenteric nodes contain many more young forms than do other nodes, and they are abundant in the sinuses. Where these young cells are found there are no plasma cells of the Marshalkó type.

There is no difficulty in discriminating Marshalkó plasma cells and the degenerating forms from lymphocytes of all types. It has been a rule to reserve the name plasma cell for these mature forms. However, many workers have included abnormal types of cells in this category. This should not be done unless these cells have one or several of the characters of true plasma cells, and show by proximity that they are developing into more mature typical forms. The differential characters of the young cells of the lymphocytic and plasma cell series are definite though not marked. This, together with the fact that few plasma cells developed in organs rich in lymphocytes after direct stimulation of those organs, makes it clear that plasma cells are not derived from lymphocytes. With a separation of the young cells into two classes the maturation cycle of the plasma cell is complete.

SUMMARY AND CONCLUSIONS

As result of finding numerous plasma cells in the omenta of rabbits injected with tuberculo-protein, a method to induce the production of large numbers of these cells has been discovered. The tissues in which they were pronouncedly increased were the subserosal connective tissues of the omentum, body wall, and intestinal wall.

The precursor of the plasma cells is a primitive connective tissue cell. As this cell develops into the typical Marshalkó plasma cell there is a progressive increase in the basophilia of the cytoplasm, the nucleus becomes eccentric, a condensation of the chromatin occurs near the nuclear membrane, and there is a loss of the nucleoli. At the time when the nucleus assumes the eccentric position, the clear area appears in the center of the cytoplasm. The early cells are capable of reproducing themselves by mitosis, while the typical mature cells divide by amitosis.

The mature plasma cells often have muddy, spongy cytoplasm which contains acidophilic or hyaline granules as the cells grow old or begin to degenerate. The cells with granules or hyaline bodies usually have pycnotic or fragmented nuclei. These cells are the final stage reached by some plasma cells. Others, when degenerating, show vacuoles and signs of senility. Those with the granules and hyaline bodies are the so called Russell body cells.

Plasma cells developed in greatest numbers after our largest injections of tuberculo-protein. The differentiation into young, mature, and senile forms was most clearly recognizable when some days had been allowed to elapse after the last large injection of the stimulating agent.

A description of the plasma cell as viewed supravitally has been given. The cells are met in the blood stream as well as in the tissues. They are characterized by their deep yellowish gray cytoplasm, indistinct eccentrically placed nuclei, and large numbers of mitochondria.

The plasma cells differ from lymphocytes, in that they did not develop in large numbers after direct stimulation of the lymph nodes with tuberculo-protein. The young plasma cells also differ in morphology from the young lymphocytes. When plasma cells were found in the lymph nodes they were in the connective tissue cords.

The plasma cell is a definite entity, having a maturation cycle. It is stimulated to great proliferation by certain toxic irritants.

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EXPLANATION OF PLATES

PLATE 30

FIG. 1. Section from body wall of Rabbit R 1168 which received twenty-five intraperitoneal injections of 10 mg. each of alkali-soluble protein. It shows many young plasma cells; two are primitive connective tissue cells (Arrows), many have eccentrically placed nuclei, and the chromatin of most of the nuclei shows a beginning condensation near the nuclear membrane. Hematoxylin and eosin. × 1,000.

FIG. 2. Section from omentum of Rabbit R 1102 which received twenty intraperitoneal injections of 20 mg. each of 304 protein. It shows plasma cells of the typical Marshalkó variety. The arrow points to a cell assuming the characters of the mature form. Hematoxylin and eosin. × 1,000.

FIG. 3. Section from the wall of the large intestine of Rabbit R 1153 which received twelve intraperitoneal injections of 10 mg. each of alkali-soluble protein.

It shows degenerating plasma cells, including some cells which contain Russell bodies. Arrows labeled A point to almost typical Russell body cells, while Arrows B indicate cells with acidophilic granules. These cells are less mature than the typical Russell body type. Hematoxylin and eosin. × 1,000.

FIG. 4. Omental spread taken from Rabbit R 1166 which received three intraperitoneal injections of 20 mg. each of 304 protein. It shows four plasma cells, one of which has about it the fibrils of a fibroblast. It was first studied in supravital neutral red and Janus green, then fixed in methyl alcohol and stained with methylene blue and Giemsa. × 1,000.

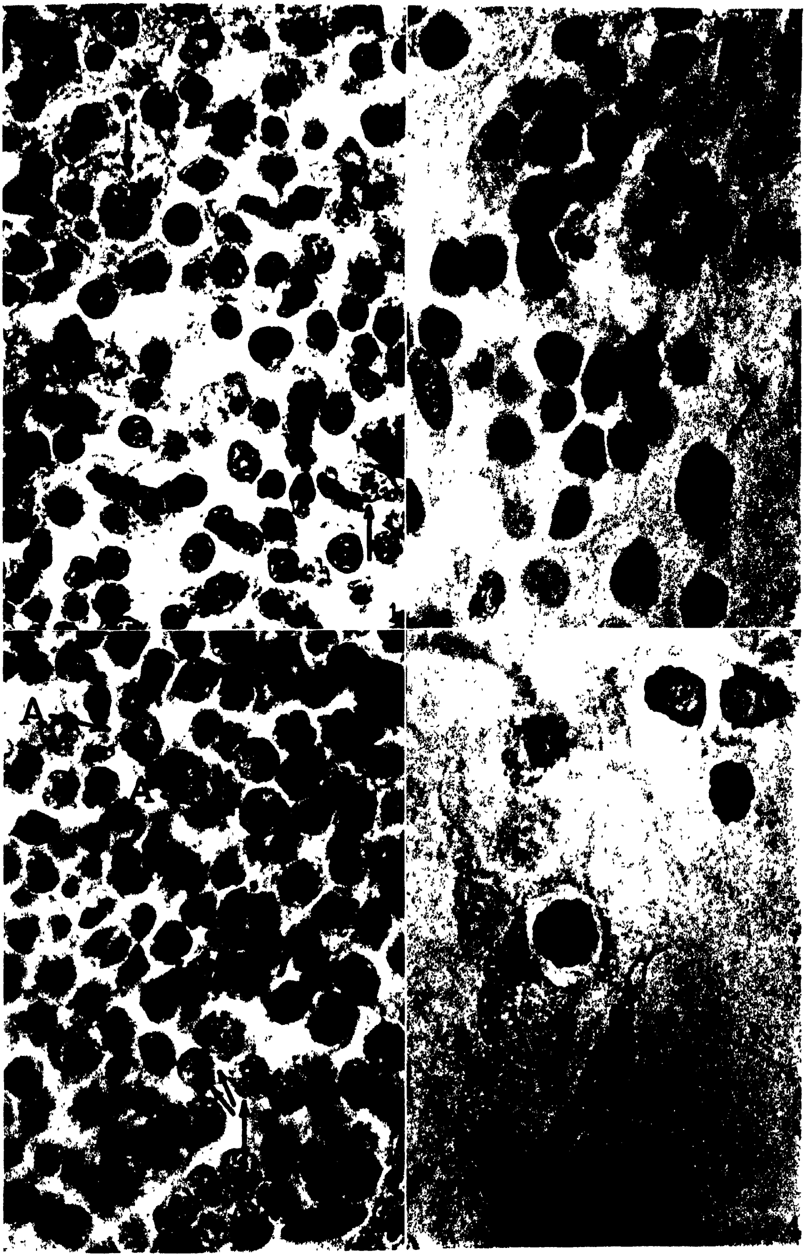
PLATE 31

FIG. 5. Section of omentum of Rabbit R 1102 which received twenty intraperitoneal injections of 20 mg. each of 304 protein. It shows a focus of typical plasma cells of the mature form. Hematoxylin and eosin. × 1,000.

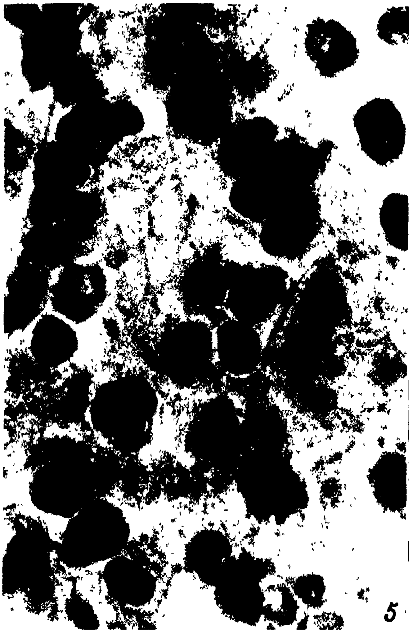
FIG. 6. Section of omentum of Rabbit R 1168 which received twenty-five intraperitoneal injections of 10 mg. each of alkali-soluble protein. This figure shows plasma cells in several of the phases of maturation. One cell in the lower right corner is budding a smaller cell, a mitotic figure is present above the center to the left, and there are several young cells scattered through the figure. Hematoxylin and eosin. × 1,000.

FIG. 7. A portion of the same section shown in Fig. 1. It has many young plasma cells, one (Arrow) just beginning to differentiate from the primitive type. In the background are young connective tissue cells and clasmatocytes. Two large pale clasmatocytes are plainly seen near the upper right border. Hematoxylin and eosin. × 1,000.

FIG. 8. Section of the same material as Fig. 5, but from a less cellular part. It shows scattered plasma cells, some more mature than others, and several long fibroblasts. Hematoxylin and eosin. ×1,000.



(Miller: Histogenesis of plasma cells)



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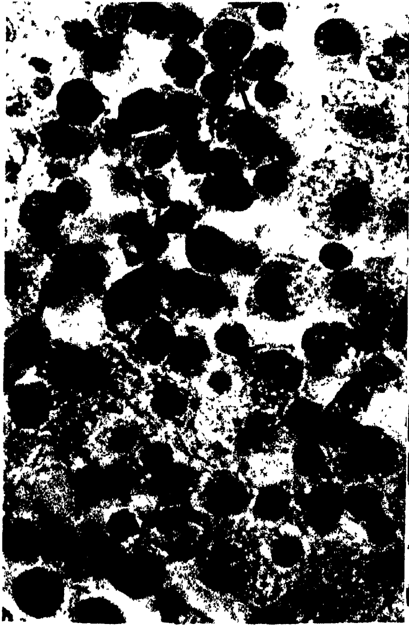

(Miller: Histogenesis of plasma cells)

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STUDIES ON THE BLOOD CYTOLOGY OF THE RABBIT

VIII. THE BLOOD OF NORMAL RABBITS AS AN INDEX OF THEIR RESISTANCE TO A TRANSPLANTABLE NEOPLASM

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This paper reports an investigation in connection with the study of animal constitution as a factor in susceptibility and resistance to disease (1). Blood was the constitutional factor studied because of the basis for work furnished by preceding papers which deal with the distribution, the spontaneous variations, and the interrelationships of the blood cells in the normal rabbit (2–7). A malignant neoplasm was chosen as the disease to be followed because of the knowledge already acquired concerning the blood reaction in rabbits inoculated with the tumor (8).

Two fundamental considerations underlie the present statistical investigation of the relationship between the blood cytology of normal rabbits and their susceptibility to this malignant disease. First, the malignancy of the transplantable neoplasm as observed in this laboratory varies from season to season and year to year, both as concerns groups of animals and among the individuals of the groups (9–11). Second, the cellular elements of the blood of normal rabbits vary seasonally and yearly, and appreciable differences in the cell formula occur both between groups (3–7) and individuals (2).

Material and Methods

Data have been obtained from 2 groups of rabbits inoculated with the Brown and Pearce rabbit tumor. The first group comprised 78 rabbits which were the same as those used in the experiments on which the blood reaction to tumor inoculation was reported (8). A description of the animals and of the experimental methods employed has already been given. The animals which were used for working out the methods of correlation were distributed as follows:

TABLE I

Group	No. of rabbits	First blood examination	Last blood examination	Tumor inoculation	No. of blood examinations before inoculation*	No. of weeks in laboratory before inoculation
1	10†	Oct. 24, 1927	Nov. 16, 1927	Nov. 17, 1927	4	4½
2	5	Dec. 5, 1927	Jan. 5, 1928	Jan. 5, 1928	12	4½
3	9	Feb. 8, 1928	Feb. 23, 1928	Feb. 24, 1928	4	2
4	10†	Apr. 6, 1928	Apr. 18, 1928	Apr. 20, 1928	4	2
5	10	Sept. 18, 1928	Nov. 22, 1928	Nov. 22, 1928	8	8
6	5†	Dec. 29, 1928	Jan. 3, 1929	Jan. 4, 1929	3	1
7	12	Oct. 26, 1929	Nov. 19, 1929	Nov. 19, 1929	7	4
8	12	Dec. 31, 1929	Jan. 14, 1930	Jan. 14, 1930	5	2
9	8	Dec. 31, 1929	Jan. 14, 1930	Jan. 14, 1930	5	26
Total...	78					

* In an earlier paper upon the blood cytology during the course of malignant disease (8), only those blood counts made within 4 weeks of inoculation were included. In the present paper all counts before inoculation are included without regard to the time factor.

† 1 rabbit each of Groups 1 and 4 was omitted because of a complicating nephritis; and an animal in Group 6 also because it was killed for transfer material before the end of the experiment.

A second series of 13 rabbits was used as a means of testing the accuracy of the methods developed with the first group; their distribution was as follows:

TABLE II

Group	No. of rabbits	Tumor inoculation	First blood examination	Last blood examination	No. of blood examinations before inoculation	No. of weeks in laboratory before inoculation
10	8	Sept. 17, 1930	Sept. 19, 1930	Sept. 19, 1930	2	2
11	5	Dec. 13, 1930	Dec. 13, 1930	Dec. 15, 1930	10	26
Total...	13				12	28

The hematological technique employed (2) included determinations of the total red and white cells with standardized pipettes, hemoglobin estimations by the Newcomer method, and neutral red supravital differential counts. 100 white cells were counted in each differential white cell determination. The number of blood counts made upon each group of animals before inoculation varied from 3 to

12 and they were distributed irregularly over the 1 to 10 weeks immediately preceding inoculation. The values used in this analysis are the *means* of *all counts* made upon each animal *before inoculation*.[1]

The transplantable tumor is considered to be an epithelioma. It arose spontaneously in the scrotal skin of a rabbit 10 years ago (12) and has been carried from animal to animal by intratesticular inoculation for upwards of 100 generations.

Estimation of Malignancy.—Each experiment was terminated 2 months after inoculation and all surviving animals were killed by air embolism. A complete autopsy was made on every animal with especial reference to the distribution and the character of any tumor.

For the present statistical analysis, expressions of malignancy were reduced to a numerical basis by one of two methods. The first was the outcome of the experience that 2 months after inoculation, animals can be divided into four categories with respect to death or survival, namely: "deaths," "probable deaths," "probable recoveries," and "recoveries." Animals which at autopsy revealed no evidence of tumor, either at the site of inoculation or elsewhere are designated as "recoveries." Those which at autopsy showed only a slight tumor growth, usually in a necrotic condition, such as a residual primary lesion of the testicle, a circumscribed lymph node metastasis, or small peritoneal implantations, would presumably have recovered from the disease and, therefore, these individuals have been classed as "probable recoveries." The remaining animals which showed more or less widespread metastases of living tumor at autopsy have been classed as "probable deaths." In order to give a numerical value to the malignancy in each animal, the relative susceptibilities of the four classes of animals have been simply expressed as 4, 3, 2, and 1, respectively, or in percentage terms of malignancy as follows: "deaths" 100 per cent; "probable deaths" 67 per cent; "probable recoveries" 33 per cent; and "recoveries" 0 per cent.

This method of classification was used as the basis for studying the relation between the pre-inoculation blood findings and susceptibility or resistance to the tumor. The results obtained, however, were controlled by a second numerical method of appraisal. This method consists in determining the number of organs or tissues having tumor growth as determined by autopsy observation. Neither the number nor the extent of the growth in any organ nor the character of the growth is taken into consideration, but simply the presence or absence of a tumor focus. The number of possible foci has been arbitrarily set at 20, and the distribution of animal material upon this basis is as follows: "recoveries" 0 foci, "probable recoveries" from 1 to 4 foci, "probable deaths" from 4 to 12, and "deaths" from 4 to 20 foci. This second method of classification which gives a numerical value to the actual distribution of tumor in each animal has been included as supplementary evidence of the accuracy of the first method. In general, it has been found that the analytical results obtained with both methods are in substantial agreement.

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[1] The mean values used in the present analyses differ somewhat from those mentioned in the report on the blood reaction after inoculation, as has been stated in that paper (8).

Methods of Analysis and of Recording Results.—The method of determining the relation of the blood cells before inoculation to the course of the malignant disease has been to construct a simple correlation table for each type of cell studied. The four values for tumor malignancy have been placed along the ordinate and the values of the blood cell factor under consideration along the abscissa in an appropriate number of boxes. Simple averages of the malignancy of the tumor in those cases corresponding to each box along the abscissa were then made. Owing to the inadequate number of cases in some boxes, the box means were smoothed by the formula $\frac{S_A + 2S_B + S_C}{N_A + 2N_B + N_C}$ where S_B was the summation of the ordinate values in the box to be smoothed, S_A that of the preceding, and S_C that of the succeeding box; N_A, N_B, N_C represented the actual number of cases falling in the respective boxes. The smoothed box means were then connected by lines; the result of this procedure is shown in the curves of Text-figs. 1, 2, and 3. The small numbers beside each point in the curves represent the actual number of cases in each box before smoothing was done. The vertical line down the center of each chart represents the approximate modal value obtained upon normal rabbits in this laboratory.[2]

The values in the text-figures represent an analysis of the entire series of 78 animals without regard to individual experimental groups, time of inoculation, or other factors. An analysis of this series of animals group by group by a thoroughly satisfactory statistical method is virtually impossible, owing to the small number of animals in each group. But though the specific degree of relationship by groups cannot be obtained, it is relatively simple to show that such a relationship does or does not exist. Simple correlation coefficients were calculated for each of the 9 groups between the blood cell values before inoculation and the numerical estimate of malignancy, using both methods of estimating the tumor malignancy; the results are shown in Table III. Correlations were also made upon the 2 additional groups of 13 rabbits previously referred to; the results are also shown in Table III under the headings of Groups 10 and 11.

For our present purpose, the pre-inoculation blood cell values for each animal in the analysis given in Table III have been combined and are expressed by a single numerical value which is considered to represent "mean susceptibility." The reasons for expressing the entire 15 pre-inoculation blood cell values as a single value are as follows: first, the technical inaccuracy of the mean of any particular blood cell value may be great; second, a single such inaccuracy might materially affect the correlation coefficient in groups of less than 10 counts; and finally, the mathematical processes involved in handling multiple curvilinear relationships are confusing to the non-statistical reader.

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[2] Since the paper upon distributions (2) was published, a definite trend in certain classes of cells has occurred, necessitating slight changes in the modal values for total white cells, eosinophiles, and lymphocytes.

The method by which the entire 15 pre-inoculation blood cell means were combined into a single numerical value may be summarized as follows: first, the mean malignancy of the tumor for the 78 rabbits as expressed in Text-figs. 1, 2, and 3 was found to be about 58 per cent; second, an imaginary horizontal line was drawn through each chart in the text-figures at the level of 58 per cent; third, those points in the smoothed curves which fell above this imaginary line of 58 per cent were said to belong to boxes representing susceptible animals; fourth, any animal which for any given blood cell factor had a pre-inoculation mean falling into a "susceptible box," was said to be susceptible to tumor in so far as this particular blood factor was concerned; fifth, the total number of such susceptible factors was then determined for each animal. This total number was considered to be the numerical estimate of the susceptibility of the particular animal to the tumor. In Table III, the figures in the column under the heading "mean susceptibility" are the simple averages of the so called susceptible factors for each group of animals.

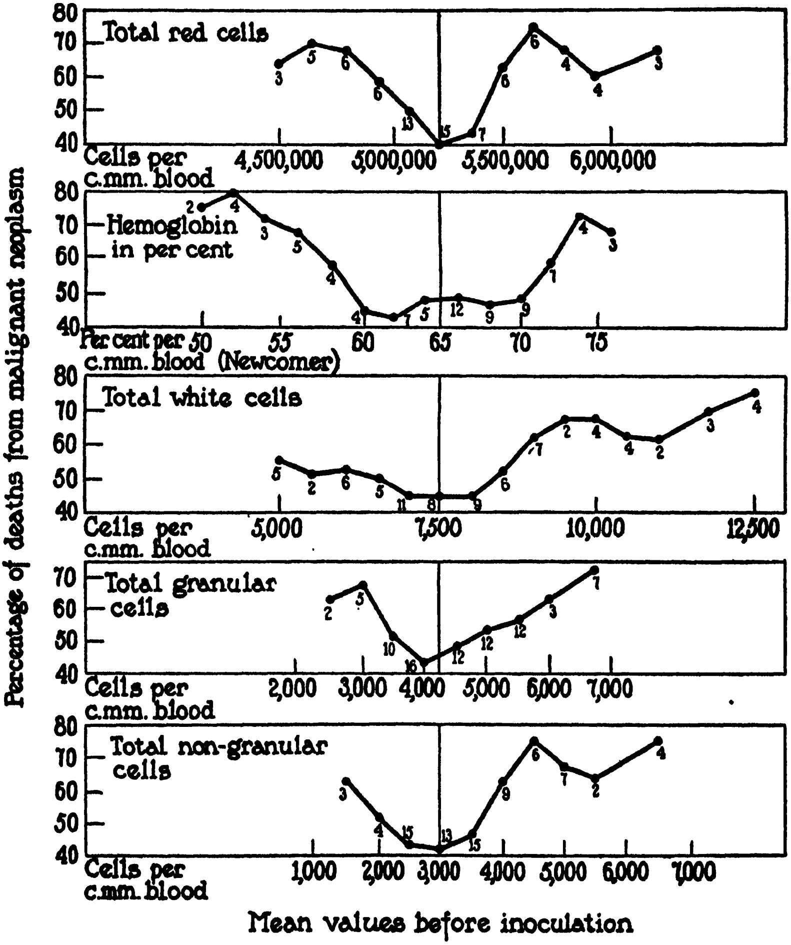
The coefficients of correlation listed at the bottom of Table III are based upon the group means given in the table and represent the relations, first, between mean susceptibility and mean malignancy, second, between mean susceptibility and mean number of foci, and third, between mean malignancy and mean number of foci respectively. A similar series of correlations upon the individual animals of each group are shown in the 3 right hand columns of Table III. Perfect correlation would be 1.000 and the absence of correlation 0.000. Neither perfect correlation nor the absence of correlation is often obtained in practice, and the coefficients obtained lie between these extreme values. The degree of correlation depends for its significance upon the number of values in the series. Since the significance of a coefficient depends upon the number of values in the series to be correlated, a correlation coefficient of +.300 obtained for the entire 78 animals would be significant while one of +.600 for 5 animals or less would not be significant. A coefficient of correlation is arbitrarily taken to be significant when the chances of its occurring by a random association of unrelated variables is less than 1 per 100. It is considered probably significant if the chances of its occurring by accident are from 1 to 5 per 100.

It should be noted that in the analysis contained in Table III, "deaths," "probable deaths," "probable recoveries," and "recoveries" are designated by the numbers 4, 3, 2, and 1 respectively while in the text-figures, these values are represented by 100, 67, 33, and 0 per cent. The use of numbers rather than percentage values in the analysis of Table III was solely for the sake of facility in making the calculations and in no way affects the results obtained.

RESULTS

The Relation of Blood Cell Values of Individual Animals to Malignancy

In Text-fig. 1 it will be noted that those rabbits with average pre-inoculation red blood cell counts varying between 5,000,000 to 5,500,000 per c.mm. were the most resistant animals to the tumor. The



TEXT-FIG. 1. The relation of the blood cytology before inoculation to the course of malignant disease.

mode, median, and mean of more than 1,000 counts on normal animals lie between 5,050,000 and 5,250,000 red cells per c.mm. of blood. The low point of tumor malignancy occurred among animals with 5,200,000 red cells per c.mm., and it would seem, then, that rabbits with normal red cell counts were the most resistant to this malignant disease. However, 40 per cent of the animals with normal counts before inoculation died from the tumor while not more than 70 per cent of animals with abnormal counts succumbed.

The values for the hemoglobin content show that the rabbits which were most resistant had mean pre-inoculation hemoglobin[3] values of between 60 and 70 per cent (Text-fig. 1). The mean, median, and mode of the normal counts already referred to lie within these limits. Again, it would seem that rabbits with abnormal values were the most susceptible to the malignant disease. Among the animals with a normal hemoglobin content, the death rate was 45 per cent and among those with abnormal values, the actual and probable death rate was 75 per cent.

The rabbits which were most resistant had mean white blood cell counts before inoculation of between 6,500 and 8,000 per c.mm. (Text-fig. 1). The mode of the normal counts was approximately 7,500 per c.mm. Animals with mean counts below 6,500 tended to be slightly more susceptible to tumor than those within modal limits. Animals with white blood cells of more than 9,000 were definitely more susceptible than those with low and normal counts. The highest susceptibility of 70 to 75 per cent occurred in the 7 animals with total white cell counts of approximately 12,000 per c.mm.

Normal mean total granulocyte counts before inoculation were in general associated with resistance to the tumor while both high and low granulocyte counts were associated with greater susceptibility (Text-fig. 1). The rabbits which were most resistant had mean pre-inoculation granulocyte counts of between 3,750 and 4,250 cells per c.mm. These values coincide with the modal point for normal animals.

The total non-granular cells of the blood before inoculation also seemed to be related to the outcome of the malignant disease. The rabbits which were most resistant had pre-inoculation values of between 2,350 and 3,750 cells per c.mm. (Text-fig. 1). The most sus-

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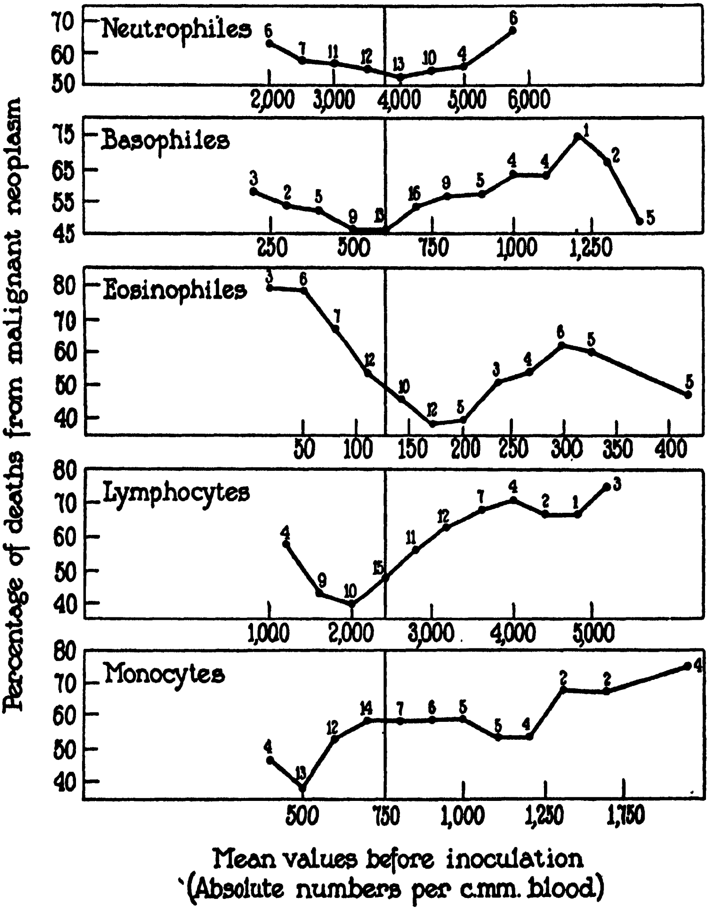
[3] The values for hemoglobin are only relative, due to the wide variation between methods of estimation, readings by different individuals, and with different instruments of the same type.



TEXT-FIG. 2. The relation of the blood cytology before inoculation to the course of malignant disease.

ceptible animals were those which had pre-inoculation values above 3,750 per c.mm. Increased susceptibility was also noted in animals with total non-granular cell counts of less than 2,250 per c.mm.

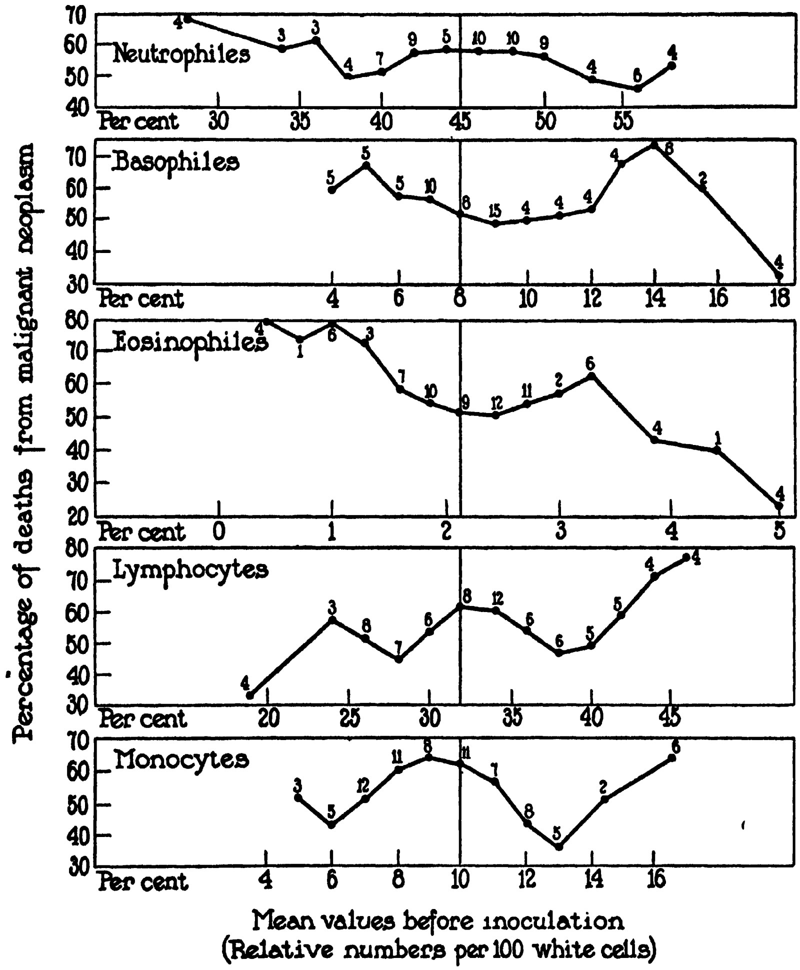
Again, deviation from the modal value seemed to be associated with lessened resistance.

Text-fig. 2 illustrates the relation of the various classes of white cells to the outcome of the malignant disease. The absolute numbers of cells per c.mm. of blood are given. It can be seen that, in general, rabbits with normal or approximately normal neutrophile, basophile, and eosinophile counts were more resistant than those with abnormal values; but it should be noted that in the case of extremely high basophile and eosinophile values, the animals tended to be resistant. In the case of the eosinophiles, however, low values were associated with an unusual susceptibility. The reaction of the various neutrophile values was of a minor order since the difference between the most susceptible and the most resistant animals was but 10 per cent.

The most resistant rabbits had pre-inoculation lymphocyte values of approximately 2,000 per c.mm.; of the 34 animals in this class, 21, or 62 per cent, were resistant to the disease. On the other hand, only 3 of the 10 animals with mean lymphocyte values of 4,000 or more per c.mm. were resistant. There was a slight tendency for animals with lymphocytes below 2,000 per c.mm. to be in the susceptible category.

In the case of the monocytes, those animals which were most resistant to the tumor had the lowest pre-inoculation values while those which were most susceptible had the highest. An exception is to be noted in the case of 4 animals with monocyte counts of below 500 per c.mm., which succumbed to the disease. The tendency for animals with low monocyte counts to be resistant and those with high counts to be susceptible is exactly the opposite to the eosinophile findings in which high values were associated with resistance and low values with susceptibility.

In Text-fig. 3, use has been made of the mean number of each white cell type per 100 cells, that is, relative values in contrast to the absolute mean numbers per c.mm. of blood previously employed. Two contrasting types of trend in relation to resistance to the tumor are to be noted. The trend of the neutrophiles, basophiles, and eosinophiles is down, that is, the more susceptible rabbits had low relative values before inoculation while the most resistant ones had high values. The trend is very similar for the three types of granular cells although it is most striking in the case of the eosinophiles. With respect to



TEXT-FIG. 3. The relation of the blood cytology before inoculation to the course of malignant disease.

the non-granular cells, however, the tendency here shown is for low values to be associated with greater resistance and high values with susceptibility. The trend in the case of the lymphocytes is in striking contrast to that of the eosinophiles. The relative monocyte values were very irregular in their relation to the outcome of the malignant disease.

It should be noted in regard to the relative values of the various white cells, as shown in Text-fig. 3, that the most resistant animals did not have counts which fell within modal limits. This is in marked contrast to the results found for the total white cell counts and those for the total granular and non-granular cells, the hemoglobin percentages, and the red cell counts, and to some extent, it is in contrast to the values for the absolute numbers of the various types of white cells (Text-figs. 1 and 2).

TABLE III

Mean Susceptibility as Determined from the Blood Cytology before Inoculation, Correlated with the Actual Course of a Malignant Neoplasm

Group	No. of animals in each group	Mean susceptibility	Mean malignancy	Mean no. of foci	r_{SM}	r_{SF}	r_{FM}
1	9	3.7	3.1	7.3	+.8974	+.6825	+.8300
2	5	2.6	2.6	5.4	+.0417	−.1379	+.8539
3	9	3.7	3.0	7.0	+.6397	+.5621	+.8669
4	9	2.3	2.4	3.6	+.5086	+.5013	+.8000
5	10	1.9	2.0	3.6	+.5770	+.7484	+.9425
6	4	4.0	2.8	7.0	+.1136	−.1199	+.8992
7	12	2.8	3.1	8.0	+.8358	+.6237	+.8548
8	12	2.4	2.6	5.8	−.1267	−.0658	+.7608
9	8	2.1	2.3	4.4	+.8017	+.8610	+.8966
10	8	3.1	2.8	5.0	+.7234	+.5757	+.9397
11	5	5.0	3.4	10.0	+.8965	+.8933	+.9863
Coefficients of correlation upon the entire 91 animals..					+.6390	+.5607	+.8858*
Coefficients of correlation upon the 11 group means.					+.8577	+.8582	+.9325

r = coefficient of correlation.
S = mean susceptibility.
M = mean malignancy.
F = mean number of foci.
* Transformed method correlations (14).

The Relation of the Blood Findings to Malignancy in Groups of Animals

In Table III which contains the analysis of the material from the standpoint of animal groups, it will be seen that the correlation between mean susceptibility and mean malignancy is greater when calculated from the 11 group means (+.858) than when calculated from the individual values for the 91 animals (+.639). In addition, the relation between mean susceptibility and mean number of foci was greater for the group means (+.858) than for the individual values (+.561). And again, the correlation of mean malignancy and mean number of foci was greater for the group (+.933) than for the individuals (+.886).

The correlation coefficients as regards pre-inoculation blood cytology (mean susceptibility) and the outcome of malignancy are comparable in magnitude with those coefficients based upon the relations between the two methods used for estimating malignancy. Both methods of estimation gave comparable results since their coefficients of correlation with the pre-inoculation blood cytology are nearly the same, namely, +.8577 and +.8582 for the 11 groups of animals and also +.6390 and +.5607 for the entire series.

DISCUSSION

No attempt has been made in this paper to give more than a brief outline of the relationships of the blood cells before inoculation to the course of the disease induced by a transplantable malignant neoplasm. The chief aim has been to show whether such relationships exist. This is the case, and it ought to be possible to predict, in the generality of cases, the eventual outcome of the disease from a cytological study of the blood before inoculation. Certain aspects of this matter and of the experimental material in relation to it will first be considered.

The Animal Material.—The use of stock rabbits of various breeds, types, and sources in these experiments was probably a benefit since a maximum number of blood variations and types of response to experimental conditions might be expected. However, the relationships encountered could conceivably be due to unknown differences in breed and type rather than to intrinsic differences in the blood formulae. In order to determine whether these relationships of pre-inoculation blood counts to resistance are dependent or independent of breed or type, other experiments with pure breeds of rabbits will have to be carried out. These are now in progress. They will serve the additional purpose of determining possible differences in cell formulae of different breeds as well as of different individuals of the same family.

The Hematological Procedure.—It is generally accepted that the numbers of blood cells in an individual animal are subject to spontaneous variations over periods of weeks or months. In attempting to relate blood findings to resistance, this feature undoubtedly plays an important rôle since an accurate blood cell formula determined upon the day of inoculation might differ significantly from one made 2, 3, or

20 weeks before inoculation. In addition, blood cell values consisting of averages of weekly determinations made during the 10 weeks preceding inoculation might differ significantly from those obtained at other intervals. The duration of the most desirable period for making the pre-inoculation counts is not known, and the appropriate number of such counts is undetermined. It seems possible, however, that with this particular tumor at least 10 counts should be made over a period of 3 weeks prior to inoculation.

The Tumor.—Any application of the results to other transplantable tumors or to the spontaneous tumors of man and the lower animals, must be made with a realization of the special material used in our experiments. From the analyses of our observations, it seems possible that a method has been evolved by means of which the cytological elements of the blood and perhaps other biometrical factors can be studied in the relation to susceptibility to transplantable tumors and other diseases. A preliminary study of such nature has already been made in experimental syphilis (16); and a more detailed report is in preparation.

In the experiments, the method of unilateral testicular inoculation was always employed. Whether the use of other sites would result in similar findings is not known. It must be borne in mind that different tumors are necessarily used as the inoculum for different experiments, and it is by no means probable that the state or condition of the tissue employed was identical in any two experiments.

The Method of Classifying Malignancy.—A satisfactory appraisal of malignancy in any given animal is admittedly difficult. The two numerical methods here employed are arbitrary and often at variance. For instance, death from inanition may result from tumor involvement of the jaw and no other tumor be found at autopsy. This might be classified as a "death" by one method or an instance of considerable resistance by another. Furthermore, an animal surviving for 2 months with widespread metastases would be described as a "probable death" while another animal dying soon after inoculation because of a single metastasis which occurred in the hypophysis would be classified as a "death." The method of counting the actual number of foci is also subject to correction since one animal with 12 foci may die in 4 weeks while another in the same group with 15 foci may live for 10 weeks. From the results of the present analyses, it seems questionable whether an animal with 10 foci is less susceptible than one with, for instance, 18 foci. It is all the more striking, therefore, that such high correlations between pre-inoculation blood findings and animal resistance have been found.

The question of the special character of the disease in relation to pre-inoculation blood cytology has also been considered, although the present material is too limited to warrant more than general suggestions. A preliminary survey, however, indicates that particular types of metastatic involvement may perhaps be associated with certain pre-inoculation blood cell values.

The Statistical Methods of Analysis.—It is quite obvious that the present problem involves multiple curvilinear correlations of a very complex sort (15). Before such an analysis can be undertaken by any of the usual methods, however, more data are necessary in order to give approximate mathematical formulae to the curves of all the various cell values in relation to resistance. Further work in the direction of normal blood cell relationships is also indicated (7). The present paper, therefore, should not be considered as an attempt to measure the *degree* of the relationships between blood cell values and resistance, nor as an attempt to express these relationships with mathematical exactness. The present data suffice merely to show the presence and the general direction of these relationships.

The Relation of the Blood Findings to Malignancy in Individual Animals

From the results, as presented, it is impossible to determine how often an animal having one blood factor indication of susceptibility will have others giving a similar indication. The following tabulation gives an idea of the frequency with which these points or values of susceptibility occurred. The number of points of susceptibility for each animal as described in the section on material and methods, are taken from the 15 blood factors representing susceptibility as given in the curves of Text-figs. 1, 2, and 3. The entire 78 animals used in Table I plus the 2 additional groups of 13 animals mentioned in Table II are classified on the basis of the number of points of susceptibility as denoted by the pre-inoculation blood cell values. The malignancy of the tumor in each class is expressed in percentage values as previously described.

TABLE IV

Points of susceptibility	No. of animals	Proportion of resistant animals
		per cent
0	6	100
1	13	93
2	22	59
3	19	26
4	12	25
5	11	27
6	5	0
7	3	0
	91	

The first fact to be noted in the above tabulation is the distribution of the numbers of animals. This distribution has a decided skew to the right, indicating that the exceptional animals of the series were more often highly suceptible than highly resistant. From this finding, it might be construed that more factors contribute toward the production of susceptibility than toward resistance.

The second point is that the blood of these animals indicated a relatively small number of points of susceptibility. Out of a possible 15 points which any animal might show, the majority had only 2 or 3 and the maximum was 7 points. A possible explanation for this finding suggests itself. The selection of normal animals for the experiments was made on the basis of weight, age, body build, and freedom from clinical signs of spontaneous disease. This implied a generally good physical condition. It might be assumed that such a healthy condition would be associated with a blood formula containing few points of susceptibility to disease, both spontaneous disease and that resulting from outside interference, such as the tumor. If this assumption is correct, it follows that rabbits with more than 7 points of susceptibility, which presumably represent a general breakdown of body resistance, would not attain the age of the animals of these experiments, or if living, would not be chosen as normal animals. This explanation is substantiated by the trends of the curves in Text-figs. 1 and 2 in which animals with normal blood values tended to be most resistant while variation either above or below normal was associated with lowered resistance to the tumor.

It is possible that susceptibility to different diseases may not be expressed by the same pre-inoculation blood formula, but by variations of a quantitative or a qualitative nature. This phase of the subject should be referred to because of the suggestion contained in the findings on the relative percentage values for the white blood cells given in Text-fig. 3. These results apparently show a certain amount of specific association in that they are independent of normal values.

The third point in the tabulation to which attention should be called is the close correlation of the number of points of susceptibility with the proportion of resistant or susceptible animals. The 6 animals which had no blood findings indicative of tumor susceptibility were entirely resistant to the disease, and the 8 animals with 6 and 7 blood factors indicative of tumor susceptibility showed little or no resistance to the tumor. In the case of the 42 animals with 3, 4, and 5 points of susceptibility, 75 per cent succumbed to the tumor. Of the 13 animals with only a single point of susceptibility, 93 per cent were resistant. The only group of animals in which it would be difficult to foretell the reaction to tumor inoculation with considerable accuracy is that with 2 points of susceptibility; 3 out of every 5 animals were resistant. This group is the largest in the series, but the succeeding one of 3 points of susceptibility is almost as large.

An index of resistance might, therefore, be devised from the above analysis in which all rabbits with 3 or more points of susceptibility are classified as susceptible and all with 2 or less, resistant. By means of such an index, 73 of the 91 animals, or 80 per cent, would be accurately classified. This accuracy can be further increased to 90 per cent for this series by the use of such quantitative biometrical methods as partial correlation. In this further analysis, account has been taken not only of the presence, but also of the degree of resistance or susceptibility as indicated by each blood factor and the relative importance of the several factors. It has been found from these calculations that certain combinations of factors seem to bear more relation to malignancy than others. These points will be discussed in subsequent papers.

The Relation of the Blood Findings to Malignancy in Groups of Animals

Probably one of the best tests of the applicability of an index of resistance derived from the values obtained on individual animals is its accuracy in representing group values. Table III represents such a test on the present material. That the coefficients of correlation upon the group values were higher than the combined coefficients upon the individual animals was to be expected; group values are less affected by errors of estimation from the standpoint of mean susceptibility, mean malignancy, and mean number of foci. The errors in calculating a coefficient based upon small numbers of animals are sometimes large as in Groups 2 and 6 with only 5 and 4 animals respectively. The very high coefficients obtained in the case of Group 11 which also had only 5 animals may be explained likewise by chance due to the small number of animals or by the fact that an especially large number of blood counts before inoculation were made.

Satisfactory coefficients of correlation depend upon accurately determined variations between individuals. In Group 8 comprising

12 rabbits, only 1 animal was a "recovery" and only 2 were "deaths." When the series came to autopsy, the distribution of living tumor in the majority of the other 9 animals was such that a classification of "probable deaths" or "probable recoveries" was unsatisfactory. It is interesting that in these particular animals, the pre-inoculation blood cell values showed few significant variations. The occurrence of such uniformity in a group with an absence of any considerable numbers of fatal or recovered cases probably explains the lack of a significant correlation. However, when these animals are considered with the rest of the 78 rabbits, either individually or as a group, their blood values are closely correlated with the course of the malignant disease. This fact is shown by the values of their group means in Table III.

SUMMARY AND CONCLUSIONS

1. The blood cytology of 91 rabbits was studied prior to inoculation with a transplantable malignant neoplasm. The following statements refer in each instance to the mean values of the pre-inoculation counts.

2. The animals which were most resistant to the malignant disease had, before inoculation, normal red and white cell counts, normal hemoglobin percentages, high eosinophile counts, and low counts of monocytes and lymphocytes. The relations of the neutrophile and basophile counts were irregular, but normal values also appeared to be associated with greater resistance.

3. The most susceptible animals were those which had, before inoculation, red cell counts above 5,500,000 or below 5,000,000 per c.mm.; hemoglobin above 70 per cent or below 60 per cent (Newcomer); white cell counts below 6,000 or above 8,500 per c.mm.; low eosinophile, high monocyte, or high lymphocyte counts.

4. No animal with any of the following findings prior to inoculation recovered completely from the tumor as determined by autopsy examination: red cells above 5,500,000 per c.mm. of blood; hemoglobin above 70 per cent; total white cells above 10,000 per c.mm.; eosinophiles below 120 per c.mm., or below the relative value of 1.5 per cent; basophiles below 400 per c.mm., or below the relative value of 6 per cent; lymphocytes above 3,600 per c.mm.; monocytes above 1,500 per c.mm.; neutrophiles above 5,000 per c.mm.; and total granular cells above 5,700 per c.mm.

In the case of each of the following pre-inoculation values, only 1 animal was completely free from tumor at autopsy: hemoglobin below 60 per cent; red cells below 4,800,000 per c.mm.; total granular cells below 3,300 per c.mm.; total non-granular cells below 2,300 per c.mm.; total non-granular cells above 3,700 per c.mm. No animal with pre-inoculation eosinophiles above 3.9 per cent, or basophiles above 16 per cent died from the tumor.

5. The blood findings before inoculation could be related to the character and outcome of the malignant disease, from the standpoint of animal groups as well as in the case of individual rabbits.

6. From the results of the experiments here reported, it seems possible to predict with an accuracy of between 80 and 90 per cent the individual resistance or susceptibility of rabbits to the tumor by a study of their blood cells before inoculation.

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THE EFFECT OF TESTICLE EXTRACT AND OF NORMAL SERUM ON A TRANSPLANTABLE EPITHELIAL TUMOR OF THE RABBIT[1]

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PLATES 48 AND 49

(Received for publication, June 23, 1931)

In the course of some experiments designed for comparison of the properties of the filtrable agents of chicken tumors with those of typical viruses, it was noted that when the tumor agent was brought in contact *in vitro* with those tissues of susceptible animals in which the tumor grows well, for example, muscle, it was adsorbed and fixed—or inactivated—while similar tissues from non-susceptible fowls or other animal species were inert in this respect (1). On the other hand, vaccine virus, which thrives in the testicle, was not inactivated by contact *in vitro* with testicle extract, but had its infectivity enormously increased by this treatment (2). This phenomenon, the enhancement of infectivity by testicle extract, has thus far proved to be universal for a great variety of viruses and bacteria (3). Work has recently been done tending to prove that the testicle extract acts by causing an increase in tissue permeability (4). The present study deals with the effect of testicle extract on the growth of a transplantable carcinoma of the rabbit, its action being contrasted with that of normal serum, which ordinarily interferes with the activity of most infective agents.

Material and Method

The Brown-Pearce epithelioma of the rabbit, a tumor having its origin in the hair sheaths of the scrotum (5), has been used for these experiments. This tumor has the peculiarity that progressive growth rarely results from inoculations in locations other than the testicle and brain. Subcutaneous or intracutaneous grafts will grow for a time but almost invariably retrogress, a fact which is difficult to explain, considering that a testicle graft often gives widespread metastases to the skin.

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[1] A preliminary report of this work appeared in *Science*, 1930, **72**, 608.

[2] This investigation was carried out by means of funds from the Rutherford Donation.

The rabbit tumor is prepared by chopping finely and then squeezing through gauze in order to get an even suspension of the cells. Before inoculation the material is diluted to two to three times its volume with Ringer's solution. The testicle extract is prepared by grinding the pure glandular tissue of rat, rabbit or bull testicle with sand and an equal volume of Ringer's solution. The mixture is centrifuged to remove the cell debris and sand. Extreme precautions against contamination must be observed throughout, to exclude incidental infection which would be enhanced by the extract.

Susceptibility of Rabbits to Intracutaneous Inoculations of the Tumor.—Preliminary tests showed that if a rich cell suspension of healthy, primary tumor or its metastases was used for the intradermal inoculations, as many as twelve such inoculations could be made in a single rabbit yielding tumors of approximately uniform size. Most of the skin tumors retrogress after 2 weeks of steady growth, during which they reach a diameter of about 3 cm.

TABLE I

Tumor cell suspension plus	No. of inoculations	Larger growth than control	Same growth as control	Smaller growth than control	No growth
Rat testicle extract	16	0	0	2	14
Rabbit testicle extract	16	0	1	6	9
Rabbit serum	32	19	10	3	0
Ringer's solution (control)	20				0

Action of Rat and Rabbit Testicle Extract and Rabbit Serum on the Rabbit Tumor

Thick cell suspensions were prepared from the rabbit tumor as described above. These were divided into four portions. To each portion was added respectively one of the following solutions: rat testicle extract, rabbit testicle extract, serum from normal rabbits or Ringer's solution. These suspensions were kept in tubes at 37°C. for from 2 to 3½ hours, during which period the tubes were shaken several times. After the incubation 1 cc. of each mixture was injected intradermally, two or more inoculations of each mixture being given to each rabbit. By this system each rabbit carried all of the tests as well as the control, thus ruling out the disturbing factor of individual differences in susceptibility. The results of five experiments, in which ten rabbits were inoculated in from eight to ten different areas each, are summarized in Table I.

As a parallel to the above experiment, eight animals were inoculated in the testicle with a mixture of tumor cell suspension and rat testicle extract. This was controlled by another group of eight rabbits inoculated in the testicle with the same cell suspensions diluted with Ringer's solution, and kept under the same conditions. The results showed some indication of inhibition of growth by the testicle extract.

The above experiments show conclusively that both rat and rabbit testicle extracts interfere with or even inhibit altogether the growth of the rabbit epithelioma in the skin. On the other hand, normal rabbit serum seems just as definitely to augment the growth of the tumor cells (Figs. 1 to 4). When the tumor and testicle extract mixture is inoculated into the testicle there is some indication of inhibition, but it is not so definite as on intradermal inoculation.

The Effect on Tumor Cells of Fresh Bull Testicle Extract and a Purified Fraction.—There is no indication in the foregoing experiments that the factor in the testicle extract inhibiting the growth of the rabbit tumor is identical with that which enhances infections and increases the permeability of tissues. In collaboration with Claude and Helmer, the fraction of testicle extract carrying the enhancing factor has been carried to a high degree of purity.[3] The entire enhancing value of 100 gm. of bull testicle has been isolated in 0.4 gm. of the purified fraction, a stable material with a very low protein content. This fraction retains the spreading power for inert particles. In the next group of experiments a comparison has been made of the action of fresh bull testicle extract with that of the purified infection-enhancing factor.

Experiment.—Five experiments were made using the same methods as described above, the test materials being the full extract and the purified fraction of bull testicle. In four of the experiments the rabbit tumor and the solution were kept in contact for from 1 to 2 hours at room temperature before inoculation into rabbits, and in the fifth experiment they were inoculated immediately after mixing. The results are summarized in Table II.

Comparing the results of these experiments with those shown in Table I it will be noted that while fresh bull testicle extract has a definite inhibiting action on the tumor, it is not quite as active as the extract of rabbit or rat testicle. The length of contact before inoculation seems to be of no importance. The purified fraction gives even more pronounced results than the full extract. While this finding does not establish the point indubitably, it suggests strongly that the factor in the testicle extract which is responsible for the enhancement of infections is also active in inhibiting the growth of the rabbit tumor.

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[3] The details of this technique will be given in another paper.

Effect of Dilution of the Testicle Extract.—In order to determine the effective strength of the testicle extract the consequences of dilution were studied.

Rabbit tumor cells were kept in contact for 2½ hours with undiluted rabbit testicle extract and with the extract diluted 1:10, 1:100 and 1:1000. At dilutions of 1:100 or over the inhibiting action was no longer evident. The same result was obtained in two other experiments in which bull testicle extract was used. The findings in a typical experiment are shown in Table III.

TABLE II

Tumor cell suspension plus	No. of inoculations	Larger growth than control	Same growth as control	Smaller growth than control	No growth
Fresh bull testicle extract	8	0	0	8	0
Purified bull testicle extract	36	0	2	20	14
Ringer's solution (control)	44				0

TABLE III

Tumor cells plus rabbit testicle extract	No. of tests	Larger growth than control	Same growth as control	Smaller growth than control	No growth
Undiluted	2	0	0	0	2
1:10	2	0	0	1	1
1:100	2	0	2	0	0
1:1000	3	0	3	0	0

In two instances in which the rabbits were inoculated in the skin with a mixture of tumor cells and diluted testicle extract, the tumor did not retrogress as it almost always does when inoculated alone, but continued to grow and gave widespread metastases. This suggests the possibility that the diluted extract may act as a stimulant.

DISCUSSION

The experiments here reported show that a factor existing in rat, rabbit and bull testicle extracts strongly interferes with or altogether inhibits the growth of a transplantable rabbit epithelioma. These findings are in strong contrast with those on the influence of the same organ components upon bacterial or virus infections. Whether the factor adversely affecting tumor is the same that enhances infections and increases the area of spread of injected material has not yet been absolutely determined. However, the fact that the highly purified enhancing factor from bull testicle manifests both spreading power and an inhibiting influence on rabbit tumor makes it seem probable that the same factor is responsible for all of these manifestations. The evidence so far gathered suggests that the increased tissue and probably cell permeability (6) brought about by the active testicle extract is the mechanism behind the phenomenon. The relation of this factor to certain other activities of testicle extract, the stimulation of normal and malignant cell multiplication *in vitro* (7) and *in vivo* (8), is as yet unestablished.

SUMMARY

Extract of rat, rabbit or bull testicle prevents or retards the growth of a rabbit tumor when a mixture of the extract and a tumor cell suspension is inoculated intradermally. Similar mixtures, made with normal rabbit serum instead of testicle extract, give rise to tumors which grow with unusual rapidity. The results are the opposite of those obtained with pathogenic bacteria or filtrable viruses which are enhanced by testicle extract and generally inhibited by normal serum.

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EXPLANATION OF PLATES

PLATE 48

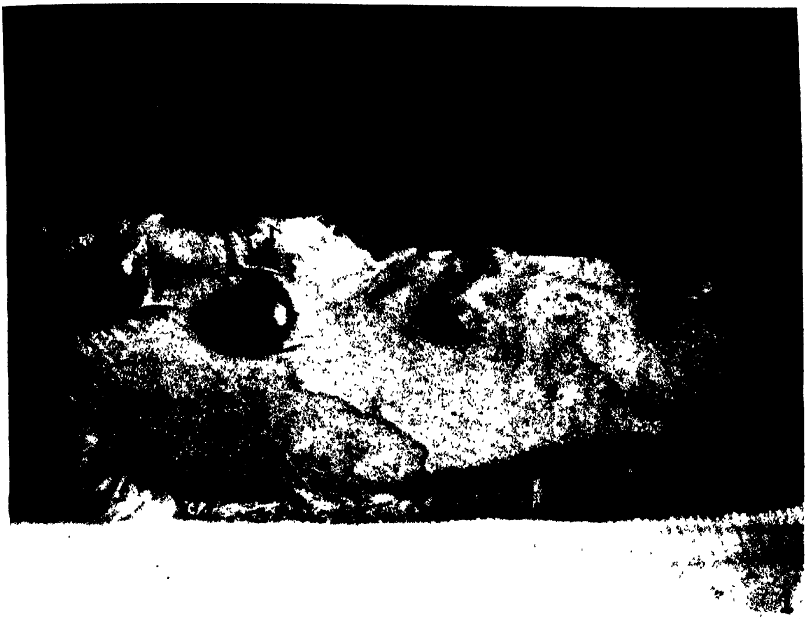
FIG. 1. *Right side.* (1) and (2) Tumors resulting from intradermal inoculation of tumor cell suspension diluted with Ringer's solution. (3) and (4) Tumors resulting from inoculation of tumor cells and testicle extract.

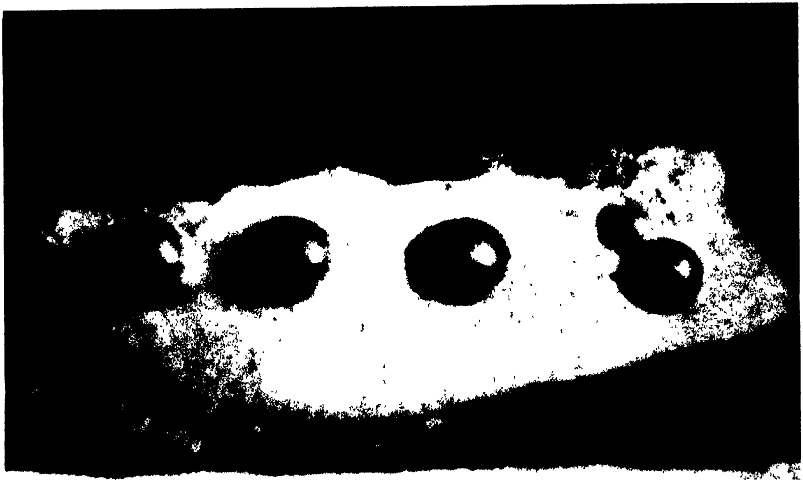
FIG. 2. *Left side.* Results of inoculation in four areas of tumor cells mixed with rabbit serum.

PLATE 49

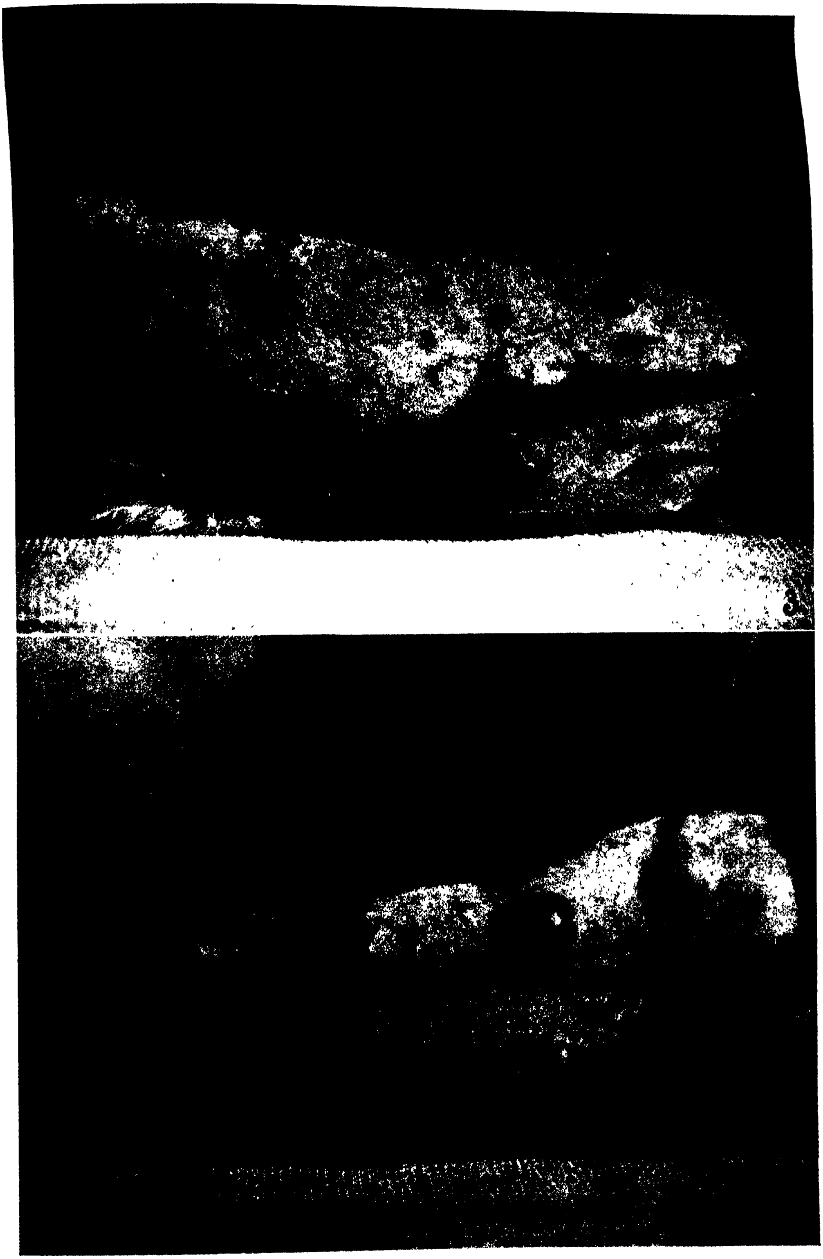
FIG. 3. *Right side.* (1) and (2) Results of intradermal inoculation of tumor cell suspension + rat testicle extract. (3) and (4) Results of inoculation of tumor cell suspension + rabbit testicle extract. (5) and (6) Results of inoculation of tumor cell suspension + Ringer's solution.

FIG. 4. *Left side.* Results of inoculation of tumor cell suspension + rabbit serum in the four areas.





(Duran-Reynals: Effect of testicle extract on tumor)



(Duran-Reynals: Effect of testicle extract on tumor)

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THE GRADIENT OF VASCULAR PERMEABILITY

IV. THE PERMEABILITY OF THE CUTANEOUS VENULES AND ITS FUNCTIONAL SIGNIFICANCE

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PLATES 50 TO 53

(Received for publication, June 27, 1931)

The recognition that permeability increases progressively along the capillaries of voluntary muscle (1) has led us to study the state of affairs in other organs. An exceedingly pronounced gradient is demonstrable in the skin of the frog, and the small cutaneous venules are even more permeable than the adjoining portion of the capillaries (2). Because of the importance of mammalian skin in water storage and regulation, we have proceeded to study this organ. The skin of the albino mouse was chosen for the purpose because the spread of dyes from the blood can be followed with special ease in it.

The Escape of Dyes from the Blood into the Skin of the Body

In preliminary tests vital dyes of known diffusibility were injected into a tail vein of unanesthetized animals which were decapitated shortly after. The skin was found to color evenly with rapidly diffusible pigments, whereas with poorly diffusible ones, such as are especially suited to disclose local differences in vascular permeability, there was to be seen a deep staining of the tissue through which the small venules coursed, before any general coloration of the corium had taken place. The phenomenon was visible only when a skin flap was studied from its under side and even then ordinarily the subcutaneous fat prevented a clear view. To do away with it mice were kept on a reduced ration until they had become thin. They yielded pictures that were highly informative.

Young mice of 17 to 19 gm. were used. They had free access to water at all times and remained lively throughout the thinning period of 7 to 9 days. When the weight had fallen to 10 to 12 gm., dye was injected. In type experiments Chicago blue 6B was used in a half strength solution isotonic with the blood (a watery 17.0 per cent[1] solution mixed with an equal quantity of Locke's solution), 0.08 to 0.1 cc. being given in the course of a minute. Decapitation was done 6 to 10 minutes later, the animal was pinned on a board at once, and a skin flap everted. The hair was oiled where it was to be cut through, in order to prevent a scattering of ends on the exposed surface. With blunt scissors a first slit was made along one side in the axillary line and this was extended transversely across the upper and the lower abdomen to the other side. The flap was freed from the web-like underlying tissue with the scissors, and everted and brought in contact with a piece of microscope slide, to which its moist surface at once adhered. Special care was taken to prevent distortion or stretching. The preparation could now be inspected under the binocular dissecting microscope by the cooled light used in our previous work (1). The hair provided an underlying ground of white against which the local color differences stood forth brilliantly. Even the gentlest shaving before injection of the dye resulted in much disturbance of the local circulation, with not a few vascular areas closed off, and irregular staining.

Chicago blue 6B is a poorly diffusible dye (1), as evidenced *in vivo* by its tardy escape from the vessels and its slow secondary spread. It colors the mouse much faster than the rabbit, yet a considerable period elapses before the skin becomes evenly stained. In mice killed 6 to 10 minutes after injection the everted flap has a remarkable appearance (Fig. 1). It is everywhere stippled with small blue dots on a ground which is unstained, appearing white because of the hair beneath. The dots are 0.1 to 0.2 mm. across, 1/2 to 1.0 mm. apart, and in the midst of each ramifies a solitary, contorted, venular tree (Figs. 1, 3, and 4). Some of the corresponding arterioles still contain sufficient dye for the fact to be made out that they are interspersed between the venules with notable regularity, and that each passes to the center of an unstained area (Figs. 3 and 4). The colored dots are most widely separated in the neighborhood of the midline, where vessels in general are fewest and the staining least. Here also the muscle of the panniculus carnosus is practically non-existent (Fig. 6), whereas toward the sides it thickens, as does the connective tissue also, and some general staining is superimposed upon the dotting. In the thinned mice fat has practically disappeared from the skin, and, looking at its under side, one can make out the butts of the hairs, in short, irregular ranks of special whiteness (Fig. 3).

In animals allowed to live more than 10 minutes after the dye injection, some general staining of the corium takes place. This does not come about by an enlargement and coalescence of the colored dots. They remain discrete but are gradually lost in an even coloration, the result of an escape of dye from the capillaries everywhere (Fig. 2).

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[1] Much more of the purified material now employed is required to make an isotonic solution than of that used in our previous studies.

Conditions Localizing the Escape

The spread of Chicago blue through the cutaneous tissue after death is so slow that differences in its local distribution can be scrutinized at leisure. Under the microscope one sees on the under surface of the skin, slightly to one side of the midline, a tracery of fine, roughly parallel blue lines,—the dye-containing capillaries supplying the scanty fibres of the panniculus carnosus (Fig. 5). Focussing shows that the perivenular spots of color lie beyond, in the corium. The arterioles supplying the latter pass almost vertically to it, and so also with the corresponding venules. Between the vessels of the two sorts only capillary channels can be made out in the corium, and these but occasionally since most are empty of the dye-stained blood. Cleared preparations from mice injected with India ink gelatin mass (Fig. 5) show the vascular arrangement excellently described by Kreyberg for the skin of the back (3). Separate arterial and venous networks beneath the panniculus connect with the capillaries of this muscle sheet and send off branches that pierce it to join other, finer-meshed, networks just superficial to it. From these in turn small, solitary arterioles and venules pass at almost regular intervals to the capillary network in the corium. The staining with Chicago blue is localized to the tissue in the neighborhood of these final venules. Hence the dotting with color seen in the gross.

The singular localization of the staining suggests the probability that there are special structures for which the dye has an affinity. But microscopic sections stained with eosin and methylene blue (or hematoxylin) have failed to disclose any such. The mammary gland lies beneath the panniculus (4) and can be ruled from account. The hair follicles fail to "take" the vital dyes, their arrangement has no relation to the dotting with color, and except for them the stratum composing the corium appears to have the same general texture everywhere in the region studied (Fig. 6). The subcutaneous fatty layer had practically disappeared in our thin animals; and it does not stain, nor is it so situated as to be responsible for the localization of color. Even in well nourished mice there is almost no fat in the corium itself of the abdominal region. Furthermore the edges of the colored dots are ill defined, fading off gradually; and all dye can be forced out of the corium by gentle pressure, leaving it unstained,—facts both which indicate that it is dissolved in the tissue fluids. And finally, dotting of the same sort is produced with other dyes fitted by their rate of diffusibility to disclose it, namely, pontamine blue, trypan red, and brom phenol blue. True, the capillaries are so permeable to this last that it must be injected very rapidly and the corium examined practically at once if the dotting is to be perceived amidst an intensifying general coloration; while with trypan red the examination cannot be much deferred. But these differences are obviously dependent, like those in voluntary muscle (1), upon the degrees of diffusibility of the dye stuffs.

Can local vascular differences be responsible for the stippling of the corium with color? One such difference is very evident. The contorted, venular trees offer a far greater surface for escape of dye from the blood than exists elsewhere. But the dye is not narrowly localized next the venular wall nor does it extend therefrom secondarily to form the spots of color. These arise *in situ* and they extend considerably beyond the region in which the venular tree is situated. To explain their relatively large dimensions one must suppose some dye to have got out from the portion of the capillaries adjoining the venules. Injected specimens show that in the neighborhood of the latter the capillary web is not especially abundant, nor are the channels wider there (Fig. 5).

The Escape into the Skin of the Ear

It is impossible to watch directly the spread of dyes from the blood into the skin of the body of the living mouse. But the ear presents excellent opportunities for this.

The mouse ear offers great advantages for the study of vascular phenomena, yet it has been little utilized. Leonard Hill has made pressure experiments upon it (5); and Lehmann (6) states that he had been able to see trypanosomes in the blood passing through it. Fröhlich and Zak (7) give the only description of the arrangement of the vessels that we have been able to find; but, while adequate for their purposes, it does not provide the information necessary for ours.

Young mice of 14 to 20 gm. have more transparent ears than older ones, while in the latter, furthermore, ecchymoses of stain often develop, traceable to injuries not easily recognized beforehand. A young animal of 20 gm. injected into a tail vein with 0.08 to 0.1 cc. of half strength Chicago blue solution during the course of a minute, manifests no symptoms, but the ear becomes pale azure, with thread-like dark vessels sharply outlined under the microscope (Fig. 9). They stand forth against a background as of shimmering ground glass (the underlying fat). After 2 to 3 minutes a patchy coloration begins of the tissue about some of the vessels, and within 5 minutes this has become marked (Fig. 10). With the binocular microscope one can see that the dye has passed out into the tissue traversed by the further portion of the capillary web and the smallest collecting venules, while no staining whatever has occurred as yet in the region occupied by the proximal part of the web and the arteriolar ramifications.

Microscopic observations are best made under ether or luminal, with the mouse so arranged on its belly in a shallow trough of plasticine that the ear stands free and vessels leading to it are not pressed upon. The hair need not be removed, but the skin should be oiled with neutral paraffin oil. Under the ear at a distance of 2 or 3 mm. is fixed an ovoid or triangular plate of opaque, white glass on a dais of plasticine, and the light of the arc is directed obliquely to illumine both glass and ear. In the combination of transmitted and reflected light thus provided the spread of dyes from the blood can be readily followed. For photographic purposes the ear is momentarily flattened in oil between the white plate and a coverslip (Figs. 9 and 10). Under such circumstances, the passage of individual red cells along the capillaries is easily discerned. But even the slight pressure of the coverslip alters the rate of staining and should be avoided.

After an injection of Chicago blue the vessels do not dilate, but the stained blood gives them almost the sharpness of a woodcut. The larger ones, veins and arteries together, radiate in a fan on the upper side of the ear, breaking up near its margin into a multitude of fine branches so interlaced that here it is often difficult to determine the type of vessel from which the dye first escapes. Between the sticks of the fan, though, the situation is better defined. One sees solitary arterioles and venules (Figs. 9 and 10) with only capillaries in the tissue between, indistinct faint blue lines which bend and loop. The posterior quadrant of the ear yields the clearest pictures.

The first escape of dye, occurring 2 to 3 minutes after the injection, stains the tissue lying immediately about the least venules; but almost as it does so the adjacent tissue turns blue too, so that an irregular colored strip is formed, with fading margins and the venule at its center. There can be no doubt, however, that extravascular dye is first perceptible in the tissue immediately next the venules; but it undergoes no evident lateral spread. After 15 minutes the strips are much more intensely blue than at first and definitely larger. In the gross the ear appears brilliantly patched with blue. Each patch or strip can be seen with the microscope to be separated from the nearest dye-containing arteriole by a region of unstained tissue, supplied by capillaries only, which is often nearly 1 mm. across but averages about 1/2 mm. (Fig. 10). Such regions become diffusely stained later, but the strips of intense blue are often discernible for more than an hour amidst the deepening color.

Structure of the Ear

The precise situation of the dye can be readily determined. Stained cross-sections show that the ear has a central lamella of cartilage with a fatty layer to either side, overlain by corium and epidermis (Figs. 7 and 8). Amidst the fat on the outer surface are irregular aggregates of short voluntary muscle fibres. These form an almost continuous flaring layer near the base of the ear but further out are scattered, and in the region we have studied, near the margin, are wholly lacking. The fan of large vessels lies on the outer side just under the corium. If a patchily stained ear is cut off, seized at its base with forceps and pulled apart, it separates into two layers, the outer, thinner one consisting only of corium and epidermis with some scattered, easily recognizable fat cells and the fan of vessels. The relation of the patchy staining to these last remains unchanged, and one perceives that the coloration is limited to the corium. Or the intact ear can be cut across with a sharp razor and inspected microscopically, edge on, in paraffin oil. One sees then that the fatty tissue and epidermis have not stained, and the cartilage only where it is thickest, and there lightly and evenly. The blue patches lie entirely in the corium. Hair follicles are equally numerous in the patched and unstained regions. They have not colored. Staining is least pronounced near the margin of the ear, because here the corium is thin and the follicles numerous.

The fatty layer on the inner (under) side of the cartilaginous lamella is shallow and incomplete, and the corium appears thick in comparison with it (Figs. 7 and 8). Both are supplied by derivatives from the fan vessels which pierce the cartilage vertically here and there to ramify and form wide meshed networks beneath it. When the ear of the living animal is viewed on its inner side, arteries and veins are seen to emerge together through a brilliantly translucent partition as of minute glass beads (the cartilage), and to branch like spider angiomata or contorted dendrites. Here again a blue patching can be seen to develop after the injection of Chicago blue, and one observes that it is localized to the tissue in the neighborhood of the venules.

The ears of mice injected with a gelatin mass containing India ink show far more vessels than Chicago blue renders visible (Figs. 11 and 12). Excellent preparations can be obtained when the aorta and vena cava are clamped off above the diaphragm just before the vessels of the head are washed out and injected by way of a cannula passed up into the aorta through the left ventricle. Not only is each large vein in the fan-work accompanied by an artery, but so too with most of the secondary and even tertiary branchings. Between the sticks of the fan, however, are some regions supplied and drained by solitary vessels; but such regions are far fewer and smaller than one would expect from the study of living animals with dye-stained blood. When the ears of these are closely scrutinized, one finds many regions in which there is no blood flow, the red cells being stationary where present at all. The smaller venules from such regions are devoid of dye. Evidently circulation is not taking place through the entire vascular bed but is much restricted, as is normally the case in the ears of the dog, cat, and rabbit (8). If an India ink suspension is rapidly thrown into the blood and its advancing dark columns are followed, one sees that they go, not from the arterioles to the nearest venules, but across gaps about 0.5 mm. wide bridged only by capillaries.

No arteriovenous anastomoses such as exist in some other species (9) have been detected anywhere in mouse ears cleared after injection with gelatin mass containing India ink; nor does dye or ink injection during life disclose the presence of any, even when the distribution of these substances has been slowed by pressure on the vessels at the base of the ear. Such anastomoses, if they exist, play no part in the phenomena with which this paper is concerned.

As already stated, the main vessels of the ear are situated on its outer side. In proportion as they branch they tend to become more superficial, and the smaller ones, which supply the corium with capillaries, lie next this layer or within it (Figs. 7 and 8). Capillaries are given off also to the fat, but with these we are not concerned since the adipose tissue shows no color; and there are deep ones supplying the voluntary muscle. The muscle capillaries are easily recognizable as grids of crowded, minute, parallel vessels (Fig. 11). They are definitely less permeable to dyes than the capillaries of the corium, but are so close set and numerous that the stained blood within them causes darkenings near the middle of the ear (Figs. 13 and 14). The anterior third of the organ is thicker than the rest, more hairy, and possesses in the corium a meshwork of large, very permeable capillaries. This region we have not studied.

In vitally stained mice the arterioles can be traced to their tips because they lie in tissue that for long is unstained. Their lumen is reduced to even less than capillary size before they break up. The open capillaries are from 3μ to 10μ in diameter, averaging 6μ, the first venules 6 to 18μ, the next larger 37μ as an average, the branches into which they give 60 to 75μ, the primary branches 75 to 150μ, and the large, fan veins 100 to 175μ. Even the largest veins are notably thin-walled, and those from which staining occurs show only a single layer of endothelial cells, in ordinary cross-sections.

The capillaries of the corium are no more numerous near the venules than elsewhere, and they first undergo enlargement when entering these vessels, as can be well seen where they enter at right angles. Where the venule continues the direction of the capillary, it is impossible to say precisely where one begins and the other leaves off.

As this description brings out, the vascularization of the corium of the ear differs from that of the body only to the extent necessitated by the shape of the organ. The vessels of the ear lie much more nearly in one plane because of the general flattening. The arterioles and venules do not rise almost vertically toward the surface and branch to all sides but ramify in the direction of the margin of the ear. Hence, as vital staining takes place with Chicago blue, one sees strips of color form, not rounded dots such as appear in the skin of the body. But the difference is merely superficial. The localized staining is traceable in both cases to a special escape of dye from the blood in the region of the venules and the further portion of the capillaries. The same holds true with other dyes, pontamine blue, trypan red, trypan blue, brom phenol blue (Fig. 13), and even the very highly diffusible patent blue V (Fig. 14). All were injected in isotonic solution (1). The variety of the dyes, and the ease with which all color can be forced from the stained tissue by general pressure rule out a fixation upon the structures of the ear.

The Gradient of Vascular Permeability

The venular trees are broader and more contorted in the ear than in the skin of the abdomen, sometimes actually corkscrewed, and their numerous, fine branches are like widened capillaries. They provide a relatively great expanse of wall through which escape can take place from the blood. That the local conditions are especially favorable to this is shown by the course of the staining, as already described. Pontamine blue escapes from only the smallest of the venules, but Chicago blue, which is more diffusible, passes through the walls of the veins of next larger size as well, and trypan red, brom phenol blue, and patent blue V get out of veins of still greater magnitude.

Can the patching with dye be explained entirely by an escape from the venules, with secondary distribution through the tissue? The differing course of events with dyes of graded diffusibility provides the answer to this question.

Pontamine sky blue, the most indiffusible of the dyes mentioned, passes out of the blood more slowly than Chicago blue, the patching is more closely localized next the venular trees, and the generalized staining in which the patches eventually are lost takes longer to develop. The dye should be injected in an isotonic half strength solution (a 10.8 per cent watery solution mixed with an equal quantity of Locke's solution (10)), 0.1 cc. for a 20 gm. mouse. The relatively diffusible brom phenol blue (1) must be put into circulation quickly if sufficient is to reach the venules for local differences in the staining of the corium to be perceptible. When given gradually so much passes out from the capillaries that no intenser staining can take place further on from the depleted blood. When quickly injected (0.15 cc. of a 4 per cent watery solution for a 20 gm. mouse) some pale, general staining of the ear appears within a minute, and superimposed upon this a deeper patching round about the venular trees (Fig. 13). The patches are broader than with Chicago blue or pontamine blue, and, as in the case of these dyes, they develop *in situ*, not by lateral spread from the venules. In comparison with them the tissue occupied by the proximal capillary meshes and arterioles appears almost unstained. After less than five minutes the coloration has become uniform throughout the ear. When the very highly diffusible patent blue V (0.15 cc. of an 8 per cent solution for 20 gm. of mouse) has been thrown abruptly into the blood the ear rapidly becomes blue everywhere; but patches of intenser color develop with venular trees in their midst, despite the fact that the plasma has lost much of its dye along the capillary way (Fig. 14). After about 2 minutes the patching is lost in a general staining.

The changes are slower in etherized animals. Tests have shown that the differences with the dyes are not referable to differences in dosage.

The patching caused by the highly diffusible dyes cannot be the result of lateral spread from the venules, since it develops *in situ* and very rapidly. It is soon lost in a general staining. The capillaries are evidently permeable to such dyes throughout their length, but most permeable in their further portion.

Several facts speak for a permeability of the venule wall exceeding that anywhere along the capillary, and for an increasing permeability of the wall of the latter as the vein is neared. When pontamine blue has been introduced into the blood the tissue supplied from the proximal part of the capillary network remains unstained for about 15 minutes after a brilliant blue patching has appeared in the venular region. With Chicago blue the period is less; while with the other dyes a general staining develops within 2 or 3 minutes at most, the time varying with their diffusibility. In the case of poorly diffusible dyes (pontamine blue, Chicago blue) the local differences mentioned persist far too long to be explicable on the basis of relative amounts of vessel wall through which dye can escape; and with the highly diffusible patent blue V this factor would seem to be more than counterbalanced by a great loss of the pigment from the blood as it flows along the capillary way, a loss plainly evident in the relatively light hue of the contents of the venules,—from which vessels, nevertheless, escape is most abundant (Fig. 14). The fact has already been mentioned that the capillaries, while exhibiting great individual variation in calibre, do not increase in width on the way to the venule. Nevertheless with a dye of medium diffusibility (brom phenol blue) a graded increase in tissue staining takes place along the greater part of this way. With less diffusible dyes a similar gradation is limited to the distal portion of the meshwork; while with the very highly diffusible patent blue V the rapidly diminishing concentration of the dye as the blood flows along the capillary sufficiently accounts for the lack of a graded staining save near the venules where the opportunity for passage into the tissues is especially good. The even staining with all the dyes, that succeeds upon the patching, is sufficient evidence that there is plenty of stainable corium everywhere.

In the course of work to be reported later with Dr. McMaster and Dr. Hudack the circulation to the mouse ear was temporarily cut off without damage by compressing the large vessels at its base between a glass plate and a narrow, sausage-shaped, collodion bag. After this had been done, Chicago blue was injected, a minute or two allowed for it to distribute itself in the blood, and then the circulation was permitted to reenter the ear. Vascular relaxation had occurred as the result of anoxemia and the entire network filled at once with dark blue blood. Immediately that this happened all flow was cut off again. And now in the course of a few minutes a light staining took place with the usual patchy distribution. One could see that the plasma in the venules lost color first,—despite the relatively unfavorable ratio of wall area to vessel content,—with decolorization in the adjoining capillary region a little after. The blood in the proximal part of the capillary meshwork and in the arterioles,—which did not contract,—was still dark blue.

It seems certain from these highly various observations that the opportunity for escape from the blood increases considerably along the capillaries but is greatest in the venular region.

Most of the substances carried by the blood are more diffusible than patent blue V, the best in this respect of the dyes used (1); but the serum proteins are considerably less diffusible than pontamine blue, which stands at the other end of our series. Yet serum proteins normally pass from the blood in no inconsiderable quantity, and are found to the amount of 1 to 2 per cent in the lymph from an extremity (11). Dr. Heidelberger has kindly provided us with two colored azoalbumins, a red compound (12) and a green one, for utilization in tests of the permeability of the cutaneous vessels. The green azo compound caused vascular injury, as shown by ecchymotic staining; but the red one produced no symptoms nor evident lesions, even when concentrated to a 9 per cent solution and injected to the amount of 0.2 cc. The ear of the injected animal became gradually and evenly pink in the course of the succeeding half-hour. Since all free dye had not been removed from the preparation, and there had been abundant time for a secondary distribution of the coloring matter, the finding was uncertain in its implications. For this reason Chicago blue 6B was linked with egg albumen in our laboratory, the last trace of free dye removed, and concentration effected in isotonic saline by differential filtration under pressure through a collodion membrane permeable to the dye but not to its combination with protein. The material thus procured was far inferior in tinctorial value to Chicago blue and yielded only an equivocal extravascular coloration.

DISCUSSION

In voluntary muscle and in frog skin the arrangement of the vessels is so orderly that local differences in the opportunity for dyes to escape along them find almost diagrammatic expression in colored patterns. In mammalian skin the vascularization is far less regular yet the findings prove that the opportunity for dyes to pass into the tissues increases along the further part of the capillaries and is greatest in the region of the venules. Here poorly diffusible substances mostly escape and the passage outwards of rapidly diffusible ones takes place most readily. That intrinsic local differences in the permeability of the vascular endothelium are responsible for the gradient of vascular permeability cannot immediately be concluded, however; for the fabric just outside may conceivably act as a reinforcing wall, local variations in it conditioning exchange. This possibility has been excluded for the ear vessels by work to be reported later.

That exchange between blood and tissues may take place in part through the walls of venules has long been inferred on anatomical grounds. As far back as 1896 (13) Starling wrote that "the production of tissue fluid is limited to the region of the capillaries and small venules." Krogh has pointed out that in human skin there are practically no capillaries except those in the papillae (8), and he infers that exchange with the tissues must take place mainly through the walls of the venules which he aptly terms "giant capillaries." Lewis (14) believes that all of the small vessels are implicated in this activity and Kreyberg (3) has stated a similar view as concerns the vessels of the skin of the mouse. The significance of our venular findings lies in the evidence they afford that the opportunity for substances to escape from the blood into the tissues is not merely as good in the venules as in the capillaries but far better. In man the cutaneous venules are developed at the expense of the capillaries (15), while even in the mouse their shape, broad and contorted, is manifestly unnecessary to the mere collection of blood. The inference seem justified that the venules are differentiated for special purposes; and the known functions of the skin provide a clue to these. They demand not only an abundant vascularization, with a highly complex controlling mechanism, as Lewis has pointed out (14), but conditions which can be provided only by broad, thin-walled vessels with a slow current.

The skin is a protective organ continually subject to slight traumata and to local infections. Cohnheim (16) noted that leukocytes get out much sooner and easier from venules than from capillaries; and Tannenberg (17), extending the observation, has reported that when these cells become attached to the capillary wall, as a first step toward emigration, they are buffeted by the swift current and frequently dislodged and whirled away. This does not happen in the venules, which provide ideal conditions for emigration.

Under ordinary conditions, with many capillaries closed down, there is still a current in the venules, and here the opportunity for exchange with the tissues cannot but be best. The view has been advanced that the rapid current of capillaries is especially favorable to gas exchange with the tissues, and the slow stream of the venules to that of less diffusible substances (18). However this may be, there is no doubt that the latter, when circulating transiently like our dyes, will get out in greatest proportion, other things being equal, where the current is slow. Whether circulating antibodies and the blood proteins reaching the lymph pass out preponderantly through the walls of the venules, present evidence does not enable us to say. Doubtless many factors condition their escape besides the immediate permeability of the vascular tube.

In man the skin of the body acts importantly to regulate temperature, as it does not in furred animals. Lewis has pointed out the obvious rôle of the broad, superficial venous plexuses in this relation. And in mammals generally the skin is one of the main depots of reserve water. There are important occasions, notably after hemorrhage, when water must be mobilized rapidly for circulatory purposes. The broad, highly permeable venular channels provide opportunities for this. In a preceding paper we have described vascular arrangements in the mammalian diaphragm and the pectoral muscle of the pigeon which are obviously adapted to the elimination of waste materials from these actively functioning organs (10).

It may be asked, why skin capillaries exist at all if venules do so well? The question has special pertinence in the case of human skin with its great development of venules. But some pressure-regulating mechanism there must be; and the capillaries are known to share with the arteries in this function. They withstand pressure as the thin-walled venules cannot, because of their wide lumen. Diapedesis under pressure occurs first from the venules (16), and ecchymosis from these vessels is far more frequent than is generally recognized (19). Under circumstances of venous obstruction Tannenberg observed diapedesis taking place from the venules, then from the venous end of the capillaries, and only later from the arterial portion of these latter, despite the fact that through them the pressure was transmitted that was responsible for the lesions (17).

In preceding communications we have shown that in some regions an effective exchange with the tissues takes place through the small arterioles and venules, capillaries being few or absent where these run (1). From the point of view of exchange with the tissues no reason exists for a tripartite classification of the vessels of such regions. But in the skin the venules are not merely enlarged capillaries, functioning like them. Besides serving for exchange with the tissues and for the collection of blood they have other important purposes.

SUMMARY

The permeability of the venules of the skin of the mouse greatly exceeds that of the capillaries. A mounting gradient of permeability exists along the further portion of the latter.

The significance of these facts is discussed with relation to conditions in human skin. The cutaneous venules are differentiated for several functions besides those ordinarily attributed to them, and must be considered as specialized organs.

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EXPLANATION OF PLATES

PLATE 50

FIG. 1. Flap of the abdominal skin of a thin mouse, everted 6½ minutes after an injection of Chicago blue,—to show the dotting with color. The staining is palest along the midline. The unstained rectangular area near the upper right hand corner is the navel. × 8.

FIG. 2. A similar flap everted 45 minutes after the dye injection,—to show the generalized staining. Some deeper dots of color can still be made out. × 8.

FIG. 3. A part of the preparation used for Fig. 1, more highly magnified. The stain can be seen to lie in the tissue round about the venules. Here and there the arterial network can be perceived (A, A) but the arterioles are invisible. The interrupted white lines are ranks of hair follicles. × 17.

FIG. 4. Under side of the abdominal skin of a mouse killed 7½ minutes after the dye injection. The venules are completely hidden in stained tissue. Enough dye remains in some of the arterioles (indicated by arrows) for their situation in unstained tissue to be made out. The contrast has been emphasized with a color filter. × 17.

PLATE 51

FIG. 5. Skin of the abdomen of a mouse viewed from beneath. Specimen cleared after injection with an ink-gelatin mass. For comparison with Figs. 1, 3, and 4. From the venous and arterial networks (V and A) branches go off at intervals to the corium. Capillaries are not especially abundant in the neighborhood of the venules. × 75.

FIG. 6. Section of the everted skin of a thin mouse, from the abdomen. The end, M, was near the midline. The specimen consists almost entirely of corium. There are no recognizable fat cells, and the panniculus carnosus, which is shallow, thins in the direction of the midline. Several hair follicles have been cut through. Eosin and methylene blue. × 180.

Fig. 7. Section of the ear of a mouse near its margin. The upper side of the organ is distinguishable by the layer of fatty cells overlying the cartilaginous lamella. The gap to either side of the cartilage is an artefact. Hematoxylin and eosin. × 180.

Fig. 8. A similar specimen with ink-gelatin mass in the vessels. Hematoxylin and picric acid. × 180.

Plate 52

Fig. 9. Ear of a living mouse photographed in oil 30 seconds after an intravenous injection of Chicago blue. As yet no dye has passed out into the tissues. The arteries, thin and straight, are readily distinguishable. Some of those which course separately are indicated by arrows. ×13.

Fig. 10. The same ear photographed 5 minutes later. The solitary arteries can still be readily discerned (arrows). They pass to tissue which is unstained, whereas that in the neighborhood of the venules is already well colored. The dye has not escaped from the larger veins, however. × 13.

Fig. 11. A relatively high magnification of part of an ear cleared after injection with an ink-containing mass. Almost every artery is now seen to be accompanied by a vein. Several grids of the fine capillaries which supply muscle fibres can be made out near the lower border of the photograph (arrows). × 32.

Plate 53

Fig. 12. Mouse ear injected with an ink mass and just sufficiently cleared for the vessels of its outer side to be plainly visible. For comparison with the photographs from animals receiving vital dyes. The vascularization is much more abundant than one would suppose from the study of these latter. × 12.

Fig. 13. Ear of a living mouse photographed 2 minutes after an injection of brom phenol blue. Some general staining has already occurred. It is least marked in the regions to which the solitary arterioles run (arrows). There are many broad patches of color with venules in their midst. × 13.

Fig. 14. Ear of a living mouse 3/4 minute after injection of patent blue V. Some general staining has already taken place, which is not evident in the photograph. The blood of the venules contains so little dye that their course is difficult to make out. The solitary arteries on the other hand are clearly visible (arrows) by reason of their dye-laden contents; yet in the regions to which they run the staining is at a minimum. × 13.

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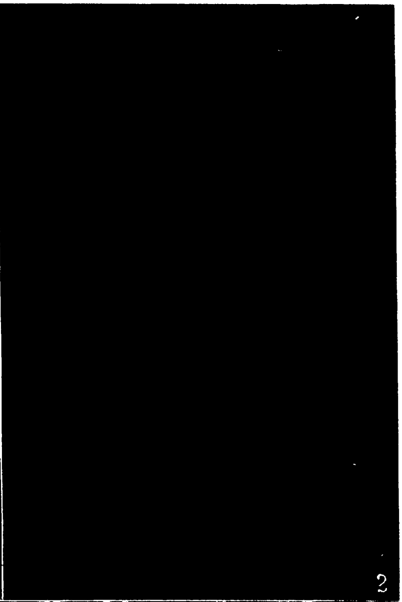

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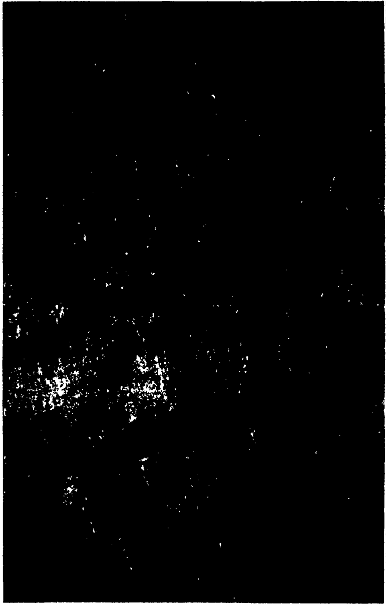

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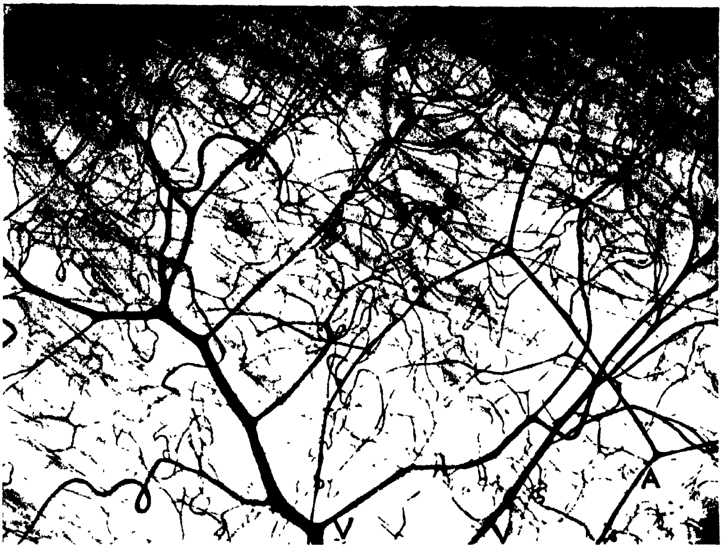
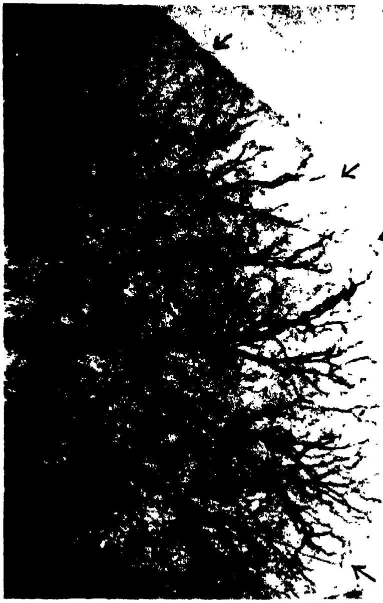
Photographed by Louis Schmidt



Photographed by Louis Schmidt

(Smith and Rous: Gradient of vascular permeability. IV)



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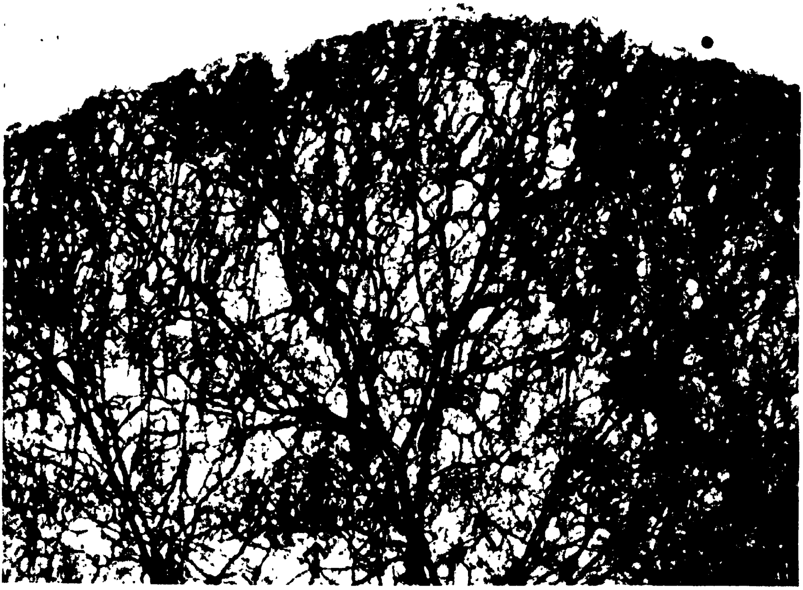

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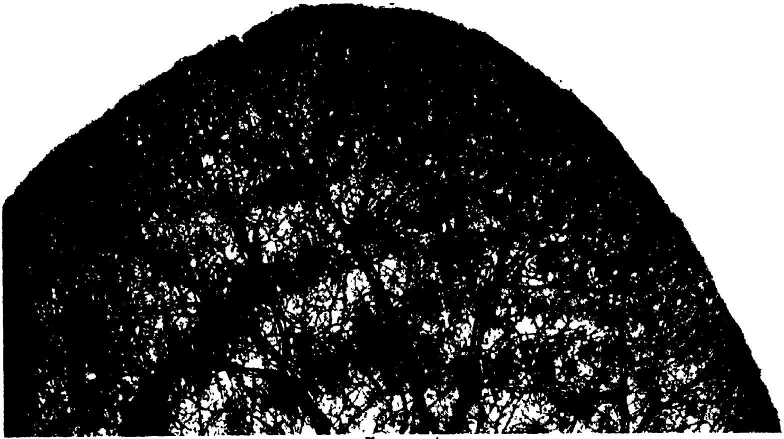
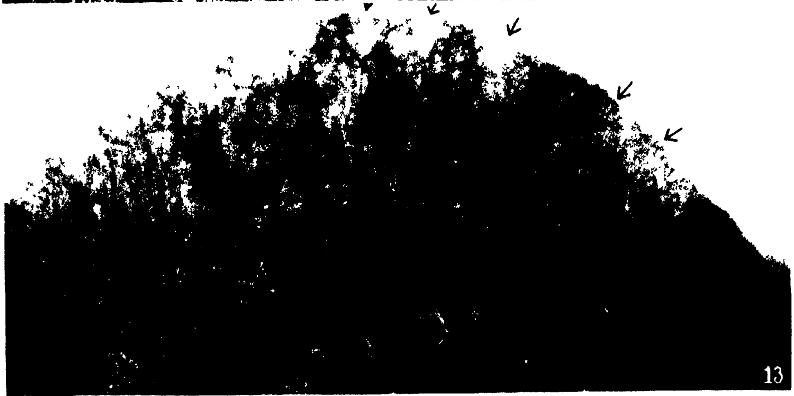
(Smith and Rous: Gradient of vascular permeability. IV)





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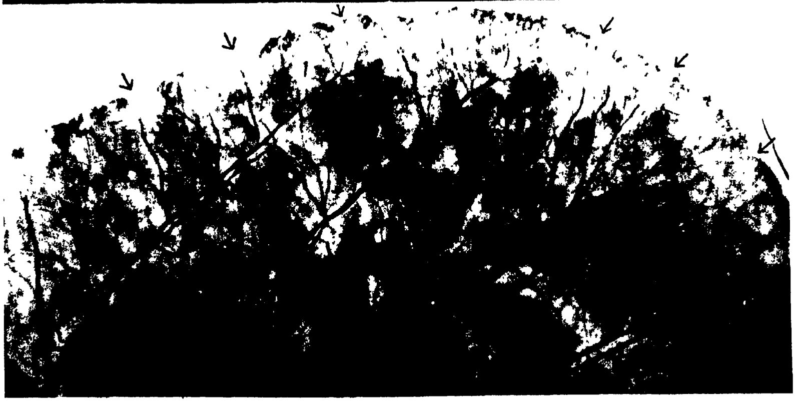

Photographed by Louis Schmidt

(Smith and Rous: Gradient of vascular permeability. IV)

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STUDIES ON THE FILTRABILITY OF BACTERIUM GRANULOSIS

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The question of whether a filtrable virus plays any rôle in the etiology of trachoma has long been a subject of investigation.

As early as 1905, Pfeiffer and Kuhnt (1) unsuccessfully attempted to produce trachoma in man with filtrates of trachomatous materials. In 1907, Fermi and Repetto (2) inoculated twenty-three humans and two apes with filtrates derived from the conjunctival material of fifty fresh trachoma cases. They used Berkefeld filters which had been tested for permeability with *B. prodigiosus*, and limited the time of filtration to 5 minutes. None of those inoculated with filtrates became infected, but the controls, injected with the unfiltered material, showed conjunctival follicles which, after 2 months, had resisted cauterization treatment. In 1910, Hess and Römer (3) excised twenty culs-de-sac of trachomatous individuals and prepared a filtrate with which they inoculated three *cynocephalus* monkeys. With the unfiltered material, they injected three other monkeys. The animals receiving the filtrate showed no reaction after several months' observation; but those which had been given the unfiltered suspension developed granulation of their conjunctivae in 4 days. Hess and Römer concluded that trachoma was not caused by a filtrable virus. As quoted by Weiss (4), Bajardi, as well as Lindner, reported negative results from inoculation of trachomatous filtrates in monkeys.

Nicolle (5) and his coworkers, in 1913, filtered trachomatous tissue suspensions through a Berkefeld filter of the "most permeable type" (presumably Type V), especially reconstructed by them for small amounts of material. The candle was impervious to *Vibrio cholerae*. The filtrate was injected into the conjunctiva of a magot. As a control, another magot was similarly inoculated with the unfiltered material. After 43 days, the first animal showed what was considered to be "typical" trachoma. This condition disappeared after a few months. The control animal exhibited granulations on the tarsal conjunctiva in 20 days. A *Macacus rhesus* monkey, also serving as a control, failed to react to the inoculations. In a second experiment, Nicolle and his associates followed the methods of the first test, save that material from two trachomatous individuals was pooled and a chimpanzee was injected with the filtrate and a magot with the unfiltered material. The chimpanzee showed granulations of the conjunctiva in 14 days; the control magot in 10 days. 50 days after the inoculation of the chimpanzee, the maximum reaction was reached, but on the 80th day the lesions had disappeared.

Trapesontzewa (6), in 1930, prepared a filtrate of the pooled conjunctival material from sixteen trachomatous patients and inoculated three blind persons. The filtration was made through a filter which permitted small amounts of fluid to be used. During 6 months' observation none of the inoculated individuals developed lesions other than an initial, slight, transitory edema. In a second experiment, the pooled and filtered conjunctival material from twelve persons with trachoma was inoculated subconjunctivally into three other individuals, including the experimenter. The only effect was a slight transitory edema, lasting not more than 36 hours. Trapesontzewa repeated this experiment twice, using material from twenty-four trachoma patients, and obtained a similar effect in both instances. No mention was made of the infectivity of the unfiltered material.

Recent work has brought out the fact that several species of bacteria are capable of passing through filters of sorts which had been supposed to exclude them. Grinnell (7) demonstrated that one has but to alter the physical conditions of a suspension of an organism in order to affect its filtrability. Cuénod (8), one of Nicolle's coworkers in the filtration experiments of the etiological agent of trachoma, says: "That trachoma can be induced experimentally by a filtrate is not opposed to the possibility of a bacterium visible microscopically."

The positive evidence that trachoma is caused by a filtrable virus rests upon the two experiments of Nicolle and his associates, in which a filtrate was active in one animal in each test. Furthermore, the "virus" of these investigators is described as being rapidly destroyed by heat at 56°C., by desiccation, and by glycerol (in 8 days). Such sensitiveness is not usual with ultramicroscopic viruses.

The following questions are unsettled (*a*) whether the incitant of trachoma is filtrable; (*b*) whether *Bacterium granulosis*, which Noguchi (9) and others have shown to be associated with trachoma, is filtrable, and, if so, whether it is pathogenic in this state, and (*c*) whether, as Cuénod and Nataf (10) suggest, a filtrable virus may be adsorbed to *Bacterium granulosis*. Because of the present uncertainty with regard to these matters, investigators believing that trachoma is due to a filtrable virus despite the greater amount of evidence to the contrary, we have made filtration tests with trachomatous materials and with *Bacterium granulosis*.

Materials and Methods

Preparation of Human and Monkey Material.—There were available for study the tarsectomized tissues from eight patients with trachoma of from 2 to 11 years' duration.[1]

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[1] For the clinical material we are very grateful to Dr. Martin Cohen of New York City.

A suspension was made of the tissue from each case by grinding for a few minutes in a sterile mortar with 2 to 5 cc. of sterile physiological saline solution. (In Cases V and VI the suspensions were made in Dunham's peptone water medium instead of in saline.) Prior to filtration, 0.2 cc. of this material was inoculated subconjunctivally into one upper cul-de-sac of each control *Macacus rhesus* monkey, and a small amount of the suspension was set aside for cultivation of *Bacterium granulosis* after the manner of Noguchi (9). Of the filtrate, prepared as described below, 0.2 cc. was inoculated subconjunctivally into each test animal and all of the remainder was used for cultures which were made by adding from 2 to 5 drops of the filtrate to tubes of leptospira medium. The tubes were kept for at least 3 weeks before they were discarded as negative.

The conjunctivae of two *Macacus rhesus* monkeys with the disease induced by inoculation of human tissues or *granulosis* cultures were used for similar filtration experiments. The conjunctivae from the upper and lower lids of one eye of each were removed,[2] and prepared for inoculation and filtration in the same manner as were the human tissues.

Standardization of Filters.—Berkefeld V filters were used. The permeability of the filters was tested with cultures of *B. prodigiosus* both before and after the filtration proper. This was done as follows:

A suspension of *B. prodigiosus* of ground glass opacity was prepared by washing growths of the organism from agar slants with sterile physiological saline solution. About 5 cc. of this suspension were introduced into the filter, under a pressure difference of 25 to 35 cm. Hg. In no test did the filtration time exceed 3 minutes. The filtrate thus obtained was inoculated into tubes of plain broth, which were incubated at 37°C. and not discarded as negative until after at least 3 days' incubation. After cleaning and resterilization, these filters were used for the experiment. Immediately afterwards they were changed to other flasks and the suspension of *B. prodigiosus* was again introduced. If, in either test, *B. prodigiosus* passed through the candle, the results of the experiment were thrown out. Almost one-half of the new Berkefeld V filters examined allowed the passage of the organisms.

Method for the Filtration of Bacterium granulosis.—Cultures of *Bacterium granulosis* from 4 to 7 days old, grown on slants of horse blood agar containing a mixture of carbohydrates (11), were employed. To each tube, 8 cc. of sterile physiological saline solution were added, the culture was scraped from the surface of the agar, and the tube shaken vigorously until a suspension of ground glass opacity was obtained. 5 cc. of this suspension were removed and put into a Berkefeld V filter, standardized as described above. The filtration was aided by a pressure difference of 35 to 40 cm. Hg and required no more than 3 minutes, usually about 45 seconds. The filtrate was cultured in tubes of leptospira medium, not more than 1 cc. being added to each tube. These cultures were incubated at 28°C. and examined daily. Before being discarded as negative, they were observed for at least 3 weeks.

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[2] Full ether anesthesia was used for all operative procedures.

Tests for a Filtrable Agent of Trachoma

Human Trachoma.—With the material from eight cases of human trachoma, seven tests were made, the suspensions from the first two cases being pooled.

Cases I and II.—Suspensions from two cases of human trachoma were pooled and filtered. The filtrate was inoculated into three *Macacus rhesus* monkeys. One of these showed a transitory, slight follicular reaction; another had a mild follicular conjunctivitis, which had disappeared in 12 weeks, and a third showed no effect. Two control animals, inoculated with unfiltered material, were used. One of these was not affected. The other showed a mild, transitory type of reaction, with a few follicles in both eyes. *Bacterium granulosis* was recovered from the unfiltered suspension. The cultures of the filtrate were negative.

Case III.—Three monkeys were inoculated with the filtrate. None of them was affected. Tarsectomy was performed on one animal and the material was cultured for *Bacterium granulosis*. The organism was not recovered.

Two control animals, inoculated with unfiltered tissue suspension, were used. One of them showed after 8 weeks a slight follicular reaction which had cleared up in 12 weeks. The other was unaffected. *Bacterium granulosis* was not recovered from the original suspension, nor was any organism recovered from the filtrate.

Case IV.—Three monkeys were inoculated with the filtrate. All remained negative. The material derived from one of them was cultured for *Bacterium granulosis* but the organism was not recovered. Of two control animals, one developed the characteristic experimental disease; the other showed a transitory, mild follicular reaction. *Bacterium granulosis* was recovered from the human case and also from the more severely infected control monkey. The culture of the filtrate was negative.

Case V.—The filtrate was tested in three monkeys. Two of them remained negative. The other animal exhibited in 2 weeks characteristic trachomatous conjunctivitis, which in 8 weeks had largely subsided. *Bacterium granulosis* was not recovered from this animal. Of two control animals, one showed no reaction; the other showed in 8 weeks a few follicles in the inoculated eye, which have persisted. *Bacterium granulosis* was not recovered from the human case. The filtrate culture was negative.

Case VI.—Two animals received the filtrate. Both remained negative. Two control animals developed experimental trachoma. *Bacterium granulosis* was recovered from the human case and from the one control monkey which was examined. The filtrate culture remained negative.

Case VII.—Two monkeys were inoculated with the filtrate. Neither of them was affected. Of two controls, one remained negative; the other developed a persistent, mild follicular reaction. *Bacterium granulosis* was not recovered from this animal. Culture of the human tissue yielded *Bacterium granulosis.* The filtrate culture was negative.

A decision as to the positive or negative character of the results was rendered difficult by the mild follicular type of reaction which some of the animals exhibited. This condition either cleared within 2 to 6 weeks, or persisted for over 12 weeks. The exact significance of this transitory type of follicular conjunctivitis is not yet clear; it may possibly have some relation to the experimental disease. Inasmuch as true experimental trachoma is progressive and persistent, only those reactions which endured for long periods of time could, on the basis of our present knowledge, be considered positive (Table I).

Monkey Trachoma.—Two *Macacus rhesus* monkeys (AA and BB) which showed the characteristic experimental disease as result of inoculation with *Bacterium granulosis* were used for this group of tests. Conjunctival tissue was removed from the animals and prepared and filtered in the same manner as with human tissue.

The filtrate from *Macacus rhesus* AA was injected into two monkeys. Neither developed granular conjunctivitis. One control animal inoculated with unfiltered tissue suspension developed experimental trachoma, and the other control showed a persistent, although mild, follicular conjunctivitis. Culture of the conjunctiva of the latter animal revealed no *Bacterium granulosis,* but that from *Rhesus* AA yielded the organism. The culture of the filtrate remained sterile.

The conjunctival suspension from *Rhesus* BB was inoculated into two monkeys. Of these, one developed characteristic trachomatous conjunctivitis, and from its conjunctiva *Bacterium granulosis* was isolated. The filtrate was inoculated into three monkeys, none of which showed conjunctival lesions. The filtrate failed to yield cultures of *Bacterium granulosis* (Table II).

A summary of the results of experiments on filtration of tissue suspensions of human and experimental trachoma reveals that only one of twenty monkeys inoculated with filtrates yielded a positive reaction, while nine of sixteen animals inoculated with unfiltered material showed characteristic trachomatous conjunctivitis. That all

TABLE I

Filtration Experiments with Human Tissue

Case	*B. granulosis* culture from human case	Unfiltered suspension			Filtered suspension				
		Monkey *a*	Monkey *b*	*B. granulosis* culture from monkey's conjunctiva*	Culture of filtrate	Monkey *c*	Monkey *d*	Monkey *e*	*B. granulosis* culture from monkey's conjunctiva
I and II	Positive (A. P.)	Negative	Negative		Negative	Negative	Negative	Negative	Negative Monkey *c*
III	Negative	Positive	Negative		Negative	Negative	Negative	Negative	Negative Monkey *d*
IV	Positive	Negative	Positive	Positive Monkey *b*	Negative	Negative	Negative	Negative	Negative Monkey *d*
V	Negative	Positive	Negative		Negative	Negative	Positive	Negative	
VI	Positive	Positive	Positive	Positive Monkey *b*	Negative	Negative	Negative		
VII	Positive	Negative	Positive	Negative Monkey *b*	Negative	Negative	Negative		Negative Monkey *c*

* Blank spaces indicate that no test was made.

TABLE II

Filtration Experiments with Monkey Tissue

Case	*B. granulosis* culture from case	Unfiltered suspension			Filtered suspension				
		Monkey *a*	Monkey *b*	*B. granulosis* culture from inoculated monkey conjunctiva	Culture of filtrate	Monkey *c*	Monkey *d*	Monkey *e*	*B. granulosis* culture from inoculated monkey conjunctiva
AA	Positive	Positive	Positive	Negative Monkey *a*	Negative	Negative	Negative		Negative Monkey *c*
BB	Positive	Positive	Negative	Positive Monkey *a*	Negative	Negative	Negative	Negative	

Macacus rhesus monkeys do not develop trachoma after subconjunctival injections of trachomatous tissue suspensions has already been pointed out by us (12) and by others. The significance of the single positive result with a filtrate remains to be determined.

Filtrability of Bacterium granulosis

In an attempt to determine whether or not *Bacterium granulosis* was capable of passing through tested Berkefeld V filters, thirty-one filtration tests were performed on ten strains of the organism. One culture was derived from one of the cases (I) mentioned above. Another was obtained from a monkey which had been inoculated with tissue from a human case not included in this series, which also yielded *Bacterium granulosis*. The remainder were old strains that had been in our possession for from 1 to 3 years.

TABLE III

Filtration of Bacterium granulosis

Strain	Berkefeld filter	Suspension fluid		Strain	Berkefeld filter	Suspension fluid		Strain	Berkefeld filter	Suspension fluid		Strain	Berkefeld filter	Suspension fluid	
		Normal saline	Hormone broth			Normal saline	Hormone broth			Normal saline	Hormone broth			Normal saline	Hormone broth
Alb. No. 1	V	–	+	EL	V	–		L-9	V	–		L-6	V	+	
Alb. No. 1	V	+	+	HD	V	–		L-5	V	–		L-6	V	+	
MC	V	–	+	HD	V	–		L-5	V	+		L-6	V	+	
MC	V	–	+	HD	V	–		Ariz. No. 3	V	–		L-6	N	–	
AP	V	–		HD	V	–		L-6	V	+	+				
AP	V	–		510	V	+		L-6	V	+	+				
EL	V	–		510	V	+		L-6	V	+					

Two tests each were made on three of the strains (including that from Case I) and no growth occurred in the filtrate. In the case of the two other strains, each passed through the filters once in two trials. Two more strains failed to come through in one test for each. The culture derived from the monkey was filtrable both times in two tests. One strain (L-6) passed through the filters in each of six trials. The experiments were repeated, using the last strain mentioned, together with one that had filtered once out of two tests, and one that had twice failed to pass the filters. Hormone broth was used for suspension instead of normal saline solution, since it has been found by Ward and Tang (13) to favor the filtrability of herpes and vaccine viruses; and by Grinnell (7) to facilitate the filtration of *Bacillus prodigiosus*. Two tests were made on each strain, and in all six trials the organisms were recovered from all tubes which had been inoculated with the filtrate. The strain which consistently passed the Berkefeld V filters was tested through an N filter but was not then found to be filtrable (Table III).

Less than half of the inoculated leptospira tubes of the "filtrable" strains showed a growth, in some instances only one tube of ten. Hence one may infer that very few organisms were present in the filtrate.

We have found, therefore, that four out of ten strains of *Bacterium granulosis* were filtrable through Berkefeld V candles. Only two of these strains filtered consistently, and it is evident that even in these cultures the organisms were present in the filtrate in small numbers.

Action of Filtrates of Bacterium granulosis

It has not been found possible up to the present time to pass a pathogenic strain of *Bacterium granulosis* through tested filters.

Filtrates of pathogenic strains of the organism, bacteriologically sterile, were inoculated into two monkeys, neither of which developed conjunctivitis. In these tests five control animals, inoculated with unfiltered suspensions of growths, developed the characteristic experimental disease. In another similar experiment, two monkeys injected with filtrates of *granulosis* cultures were likewise unaffected.

DISCUSSION AND SUMMARY

The evidence hitherto reported concerning the filtration of trachomatous material, and inoculation of man and monkeys with the filtrates points to the conclusion that the incitant of trachoma is not, as a rule, filtrable. Our findings confirm this view and indicate further that no virus causing the disease is adsorbed to *Bacterium granulosis*. On the other hand, *Bacterium granulosis* itself in heavy suspensions is irregularly filtrable through Berkefeld V candles, like some other bacteria (14), but it is present in the filtrates in only small numbers. When suspensions were used of trachomatous human and monkey tissues, which contain much fewer organisms than do actual cultures, *Bacterium granulosis* was never recovered from the filtrates.

The conception that trachoma is a disease caused by an ultramicroscopic virus is based on (*a*) the positive results of filtration in two animals, as reported by Nicolle and his coworkers, and (*b*) the presence of so called "inclusion bodies" in some of the cells of the lesions. One can state definitely that the evidence is now greatly against the filtrability of the etiological agent of trachoma. Furthermore, filtrability does not in itself suffice for the classification of an agent as an ultramicroscopic virus. Concerning (*b*), a vast literature has accumulated which indicates that the "inclusion bodies" of trachoma are not specific for the disease and that the bodies themselves may be bacterial in origin (15). We have not as yet found bodies of the kind characteristic of many filtrable viruses in the tissues of man or of monkeys with the experimental disease.

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HEXOSEMONOPHOSPHATES

SYNTHETIC ROBISON ESTER

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We have previously described[1] the synthesis of a glucosemonophosphate, to which we assigned the structure of glucose-6-phosphate, which was similar in most properties, including enzymic fermentation, to the Robison monophosphate which occurs in enzymic fermentations. Later we showed[2] that the acids prepared from the Robison ester and our ester were similar, and that the lactone formation from them followed the same course. Our ester differed from the Robison only in the melting point of its osazone and in the rotation of the barium salt. Robison has now shown[3] that the melting point of the osazone of his ester was incorrectly reported, the osazone being in fact identical with that from the Harden-Young and Neuberg esters which is what we had found for the osazone of our synthetic ester.

Purification of our earlier products had been found difficult because of a highly colored impurity which contaminated the brucine salt and which was not removed on further crystallization. We now find that by avoiding the use of charcoal this substance is present in much smaller amount and can be removed by recrystallization. A portion of brucine salt crystallized three times from 90 per cent methyl alcohol, as used by Robison for his ester, on reconversion to the barium salt, gave a product with a rotation of $[\alpha]^{25}_{5461} = +20.2°$. Robison found for his most highly purified specimen a rotation of +21°.

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[1] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **89**, 479 (1930).

[2] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **91**, 751 (1931).

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For further confirmation of the structure of glucose- and fructose-6-phosphates we have attempted the synthesis of glucose-6-phosphate from 1,2,3,4-tetracetyl-*d*-glucose which presumably has only position 6 open.[4] Phosphorylation of this product has been already performed by Helferich and du Mont[5] but their method of isolation led to the securing of tri(1,2,3,4-tetracetyl glucose)-6-phosphate and tri-glucose-6-phosphate which were of no interest to us. We modified the methods of phosphorylation and isolation, and did in fact obtain some material possessing the properties of a monophosphate. Moreover, this product gave in good yield, the same phosphosazone as that from the Harden-Young ester. However, the yield of monophosphate from the tetracetyl glucose was very low, and the reaction was peculiar in that an unusually large proportion of barium monophosphate was formed along with the barium salt of the ester. In this connection it must be remembered that the 1,2,3,4-tetracetyl glucose can be isomerized[6] with very mild alkaline treatment to a tetracetate whose structure is not yet certain.[7] For these reasons this method of approach cannot, for the present, be considered to have yielded a conclusive confirmation of the view that the Robison ester is glucose-6-phosphate.

Our intention is to prepare next the 1,2,3,5-isodiacetone of Ohle, which has only position 6 open,[8] and to phosphorylate it to secure a glucose-6-phosphate, the structure of which will be certain.

In the meantime, it seems certain that our synthetic product is the same substance as the naturally occurring aldosemonophosphate or Robison ester. Where this ester is needed for biological studies, the synthetic product is apt to be more easily prepared than the natural, and for many purposes the crude synthetic barium salt could be used without further purification.

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EXPERIMENTAL

Diacetone Glucose—The method used by Levene and Meyer[9] for diacetone galactose has been modified somewhat and utilized for the preparation of diacetone glucose. Although the yield is only 50 per cent of the theoretical, still from the point of view of time and expense the method is a considerable improvement.

To 2 liters of acetone (U.S.P.) add 20 cc. of sulfuric acid (D, 1.84), 250 gm. of anhydrous copper sulfate, and 250 gm. of glucose (cerelose, Corn Products Company, was used) in this sequence, and shake or stir the mixture for 24 hours. Filter with suction to remove copper sulfate and unchanged glucose, and neutralize with powdered calcium hydroxide. Refilter and decolorize with charcoal. Concentrate to a thick syrup under reduced pressure and extract repeatedly with boiling heptane. Decolorize with charcoal and allow to crystallize. Yield, 155 to 165 gm.

Monoacetone Glucose—The method of Coles, Goodhue, and Hixon[10] is used with slight modification.

To 600 cc. of ethyl acetate add 6 cc. of nitric acid (D, 1.42), 3 cc. of water, and 75 gm. of diacetone glucose. Immerse in a steam bath until the mixture boils, and then keep boiling for 2 minutes. Cool in running water with stirring, filter off the monoacetone glucose, and wash with more ethyl acetate. To the washings and mother liquor add ethyl acetate to 600 cc. total, 6 cc. of water, 3 cc. of the concentrated nitric acid, and 75 gm. of diacetone glucose. Heat as before. This procedure may be repeated indefinitely and serves to decrease the amount of ethyl acetate required. After hydrolyzing the last batch of diacetone glucose, add the usual proportion of water and reheat in order to hydrolyze any unchanged diacetone glucose in the mother liquors.

In recrystallizing the monoacetone glucose from ethyl acetate it is best to add enough powdered calcium hydrate to neutralize the acid which adheres to the crude monoacetone. Add calcium hydrate and charcoal to the boiling ethyl acetate solution, filter with suction, and cool. The monoacetone is obtained as a colorless, almost pure product after one crystallization.

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[9] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **92**, 257 (1931).

[10] Coles, H. W., Goodhue, L. D., and Hixon, R. M., *J. Am. Chem. Soc.*, **51**, 523 (1929).

6-Glucosephosphate from Monoacetone Glucose—The procedure is essentially that used previously; the change in time of phosphorylation increases the yield a little and gives a purer product.

The isolation is somewhat improved.

Dissolve 20 gm. of monoacetone glucose in 125 cc. of dry pyridine, cool to −35°, and add, in several portions, a solution of 8.0 cc. of distilled phosphorus oxychloride in 25 cc. of cold dry pyridine, recooling the mixture to −35° before each addition. After the oxychloride is all added, allow the solution to warm slowly to −20° and then place in an ice-salt bath at −10° to −12° for 1 hour. Add chipped ice and ice water, and make alkaline to phenolphthalein by the addition of a warm saturated solution of barium hydroxide. Without filtering, transfer to a distilling flask and remove the pyridine under reduced pressure, adding more water if necessary. Dilute to a volume of 105 cc., add 45 cc. of 5 N sulfuric acid, keep at 80° for 1½ to 2 hours,[11] and then cool. Without filtering, add a suspension of precipitated silver carbonate until all the chloride is removed. The mixture should still be acid to Congo paper, and more sulfuric acid should be added if it is not. When the solution is chloride-free, filter with suction. Suspend the precipitate in 250 cc. of 0.1 N sulfuric acid, stir well, and refilter. Saturate the filtrate with hydrogen sulfide, aerate to remove entirely the excess hydrogen sulfide, make alkaline to phenolphthalein with a warm saturated solution of barium hydroxide, and filter with suction. After concentrating under reduced pressure to about half volume, refilter, using charcoal, and continue the concentration until the volume is about 30 cc. If turbid, refilter with charcoal. Precipitate with an equal volume of 95 per cent alcohol, filter off the precipitate, and wash with 50 per cent alcohol. Redissolve in water. Concentrate under reduced pressure to the same volume as before, refilter with charcoal, and reprecipitate. Wash and dry as usual. Yield, 13 to 15 gm.

The product, as thus prepared, is a white colorless powder, analyzing well for the barium salt of a hexosemonophosphate, and having an optical rotation of $[\alpha]_D^{25} = +14°$ to $+15°$. It gives with the greatest ease the phosphosazone which melts at 150–151° and has the optical properties of the osazone of the Harden-

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[11] Josephson, K., and Proffe, S., *Ann. Chem.*, 481, 91 (1930).

Young ester. The sodium salt of this synthetic ester is fermented by zymin at the same rate as is the naturally occurring Robison monophosphate and the initial rate is the same as that of glucose.

For purification, the barium salt prepared as above was converted to the brucine salt and this was recrystallized three times from 90 per cent methyl alcohol. The use of charcoal at this stage had to be avoided as a colored impurity was formed which could not be removed by further treatment. The three times crystallized material analyzed for the dibrucine salt of a hexosemonophosphate.

A portion of the pure brucine salt was reconverted to the barium salt and this was precipitated, washed, and dried as usual. The analysis corresponded to the barium salt of a hexosemonophosphate.

4.145 mg. substance: 23.475 mg. ammonium phosphomolybdate (Pregl).
0.100 gm. " : 0.0573 gm. $BaSO_4$.
$C_6H_{11}O_9PBa$. Calculated. P 7.84, Ba 34.74
Found. " 8.22, " 33.72

The rotation was

$$[\alpha]_{D}^{25} = \frac{+\ 1.29^\circ \times 100}{2 \times 3.70} = +\ 17.4^\circ$$

and

$$[\alpha]_{5461}^{25} = \frac{+\ 1.49^\circ \times 100}{2 \times 3.70} = +\ 20.2^\circ$$

Robison found 21.0° for this last value on samples of the most highly purified, naturally occurring ester. The purity of the preparation above could presumably be increased by further crystallization of the brucine salt, but it is quite pure enough for most purposes.

Glucose-6-Phosphate from 1,2,3,4-Tetracetyl Glucose—1,2,3,4-tetracetyl-*d*-glucose was prepared from glucose through the triphenylmethyl derivative as described by Helferich *et al.*[12] and was phosphorylated as follows:

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[12] Helferich, B., Moog, L., and Jünger, A., *Ber. chem. Ges.*, **58**, 872 (1925). Helferich, B., and Klein, W., *Ann. Chem.*, **449**, 219 (1926).

4.8 cc. of redistilled phosphorus oxychloride (1.1 mols) were dissolved in 50 cc. of cold dry pyridine and cooled to about −30°. To this was added, in several portions, a solution of 17 gm. (1.0 mol) of the pure 1,2,3,4-tetracetyl glucose in 75 cc. of dry pyridine, the mixture being recooled to −30° before each addition. Heat was evolved and pyridine hydrochloride separated, but the solution remained water-white. After all the acetoglucose had been added, the mixture was transferred to an ice-salt mixture for 2 hours and was then recooled to −35°. A 10 per cent solution of water in pyridine was added until no further heat was evolved, the temperature being kept below −20° throughout. Ice was then added and finally ice water. The mixture was still water-white.

Barium hydroxide was now cautiously added to the above solution. The temperature was kept below 5° and the mixture was well stirred but at a pH of about 6 the mixture began to darken and a precipitate formed. The precipitate increased in amount and the solution became darker in color up to a pH of about 7. The addition of the barium hydroxide was continued until the mixture was alkaline to phenolphthalein, and it was then allowed to stand for a few minutes. During this time the alkalinity had decreased; hence more barium hydroxide was added to restore the pH to its previous value. This procedure was repeated until the mixture remained alkaline even after standing. It was then transferred to a distilling flask and concentrated under reduced pressure until all the pyridine was removed, water being added once or twice. The mixture was still alkaline to phenolphthalein and remained so even after warming to 35° for 15 minutes. The large precipitate was centrifuged off, the solution was filtered with charcoal, concentrated to a small volume, and precipitated with an equal volume of 95 per cent alcohol. After being washed with 50 per cent alcohol, the precipitate was dissolved in water, the solution was concentrated under reduced pressure, filtered with charcoal, and reprecipitated with an equal volume of alcohol as before. The solution and precipitation were repeated once more, and the product, which was perfectly white, was dried. The yield was only 3.2 gm., and the rotation was low; $[\alpha]_D^{25} = +11.7°$.

The analysis was only fair.

3.950 mg. substance: 23.185 mg. ammonium phosphomolybdate (Pregl).
0.0860 gm. " : 0.0490 gm. $BaSO_4$.
$C_6H_{11}O_9PBa$. Calculated. P 7.84, Ba 34.74
Found. " 8.52, " 33.53

The barium was removed from 1.3 gm. of the material and the osazone was formed in the usual fashion. The yield was 0.7 gm. which is as good as that from an equal quantity of the Neuberg or Robison ester. It melted at 149–150° and there was no lowering of the melting point when mixed with an authentic sample of the Harden-Young osazone. The rotation was $-53.4°$ in pyridine-absolute alcohol (2:3 by volume) 5 minutes after dissolving and

$$[\alpha]_D^{25} = \frac{-0.19° \times 100}{0.5 \times 1.2} = -31.7° \text{ (at equilibrium)}$$

Effect of Glucose-6-Phosphate on Induction Period—A sample of zymin prepared from a bottom yeast was used which exhibited an induction period of 90 minutes. Addition of hexosediphosphate to a concentration of 0.005 mol per liter entirely removed this induction and fermentation began immediately. With both synthetic glucose-6-phosphates, that from the monoacetone glucose and that from the 1,2,3,4-tetracetyl glucose, at concentrations of 0.005 mol per liter the period of induction was reduced to 45 minutes. This is of the same order of magnitude as the effect previously observed for the naturally occurring Robison ester at the same concentration.

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HEXOSEMONOPHOSPHATES

GALACTOSE-6-PHOSPHATE

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(Received for publication, June 24, 1931)

Through the extensive researches of Harden and others it has been shown that accompanying the enzymic fermentation of sugars, and also the enzymic processes taking place in muscle, there are large amounts of sugar phosphates simultaneously formed, and in some cases destroyed. The function of such phosphorylations and dephosphorylations has however not been elucidated. Whether some phosphate, as yet unisolated, acts as a true intermediate in the processes, or whether in some fashion these phosphates modify or condition the cleavage of the sugars, is as yet not known.

One of the ways of elucidating the function of the phosphoric esters in fermentation is to establish whether the mere phosphorylation of an otherwise unfermentable hexose endows it with this property. For example, would galactose, which is fermented by ordinary yeasts only after suitable culturing, be fermented by the yeast enzyme, if first converted into a phosphoric ester? Inasmuch as the only glucose phosphate which is readily fermentable is that in which the substitution is in position 6, the obvious ester to test would be galactose-6-phosphate. We have therefore prepared this ester and have tested its fermentability with zymin. The result was entirely negative.

A second property possessed by certain phosphorylated sugars is the ability to diminish the period of induction which is observed with certain preparations of zymin. The unsubstituted sugars are without effect. Of the phosphoric esters the hexosediphosphate has this property to the greatest degree while the Robison (glucose-6-phosphate) and Neuberg (fructose-6-phosphate) esters are less effective. We now find that galactose-6-phosphate is negative in this respect.

It is our intention to examine next the behavior of the galactose-phosphates with enzymes from yeasts which have the ability to ferment galactose.

The present observations then, serve to show that glucose-6-phosphate may not be simply an accidental product, but a substance functioning in the process of fermentation.

EXPERIMENTAL

Galactose-6-Phosphate from Diacetone Galactose—Diacetone galactose was prepared (by Dr. G. M. Meyer) by the procedure described by Levene and Meyer[1] and distilled under greatly reduced pressure.

It was phosphorylated as follows: 7.1 cc. of redistilled phosphorus oxychloride (1.1 mols) were dissolved in 75 cc. of cold dry pyridine and cooled to −30°. To this was added in three portions, a solution of 20 gm. (1.0 mol) of diacetone galactose in 50 cc. of dry pyridine, the mixture being recooled to −30° before each addition. After addition of all of the diacetone, the mixture was transferred to an ice-salt bath for 2 hours and was then recooled to −30°. A solution of 10 per cent of water in pyridine was added until no further heat was evolved and ice and then ice water were added. The mixture was neutralized with barium hydroxide, the pyridine was removed by distillation under reduced pressure, and the acetone groups were hydrolyzed off, sulfuric acid at 80° being used exactly as described for the phosphate prepared from monoacetone glucose.[2] The subsequent treatment was likewise the same, the halide being removed with silver carbonate and the excess sulfuric acid with barium hydroxide. After concentration under reduced pressure to a small volume, the material was precipitated by the addition of an equal volume of 95 per cent alcohol, was washed with 50 per cent alcohol, redissolved, concentrated, precipitated, washed, and dried as usual. The yield was 12 to 13 gm.

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[1] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **92**, 257 (1931).

[2] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **92**, 757 (1931).

TABLE I

Fermentation by Zymin of Galactose-6-Phosphate, Glucose-6-Phosphate, and Glucose

Time	Cc. CO_2 per gm. zymin			
	Blank	Galactose-6-phosphate	Glucose-6-phosphate	Glucose + sodium phosphate
min.				
4				
9	0.14	0.13	0.65	0.45
15	0.28	0.44	2.24	2.88
30	1.11	1.15	3.93	5.63
45	1.85	1.90	5.24	7.08
60	2.26	2.38	5.48	8.38
90	2.68	2.83	6.64	10.77
170	2.96	3.05	8.93	16.84

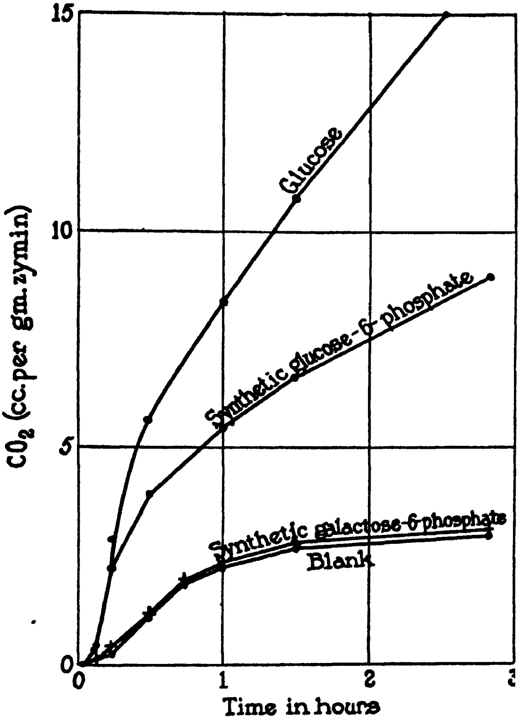

FIG. 1

The analysis corresponded to the barium salt of a hexosemonophosphate.

4.555 mg. substance: 24.595 mg. ammonium phosphomolybdate (Pregl).
0.0909 gm. " : 0.0528 gm. $BaSO_4$.
$C_6H_{11}O_9PBa$. Calculated. P 7.84, Ba 34.74
Found. " 7.84, " 34.18

and the rotation was

$$[\alpha]_D^{25} = \frac{+1.96^\circ \times 100}{2 \times 4.0} = +24.5^\circ$$

Fermentations—The apparatus and technique were identical with those previously described.[3] The esters were used in the form of their sodium salts and at concentrations of 0.06 mol per liter. Where glucose was used its concentration was 10 per cent. The induction period exhibited by the zymin was removed by adding hexosediphosphate to the extent of 0.005 mol per liter to each tube. Toluene was added in all cases. The data are given in Table I and are plotted in Fig. 1.

Effect of Galactose-6-Phosphate on Induction Period—A sample of zymin prepared from a bottom yeast was used which exhibited an induction period of 90 minutes. With the esters at a concentration of 0.005 mol per liter it was found that the induction period was entirely removed by hexosediphosphate and was reduced to 45 minutes by the synthetic glucose-6-phosphate. The galactose-6-phosphate on the other hand was slightly inhibiting and fermentation did not begin for 110 minutes.

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[3] Raymond, A. L., and Levene, P. A., *J. Biol. Chem.*, **79**, 621 (1928). Raymond, A. L., *J. Biol. Chem.*, **83**, 611 (1929).

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γ-GLUCOSIDE OF 3-METHYL-*d*-GLUCOSE

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(Received for publication, June 24, 1931)

INTRODUCTION

The present communication presents part of the work on the general problem of the influence of partial substitution on the rate of glucoside formation and on the ring structures of the glucosides that are formed. The specific interest of 3-methyl-*d*-glucose lies in the fact that this sugar is incapable of forming a propyleneoxidic glucoside. Thus, should this sugar form a γ-glucoside under the same conditions as unsubstituted *d*-glucose, then the propyleneoxidic structure is excluded for the γ-glucosides. Also, if the general conduct and the rate of glucoside formation of the two γ-glucosides are identical it will be warranted to conclude that the ring structure of the two γ-glucosides is identical. Actually, 3-methyl-*d*-glucose formed both a γ and a normal glucoside, and, moreover, the rate of formation of the γ form was essentially the same as that of the γ-glucoside from *d*-glucose. This evidence alone might suffice to exclude the propyleneoxidic structure for the γ-methylglucosides, but for greater rigor it was deemed desirable to compare the tetramethyl-γ-methylglucosides obtained from *d*-glucose and from 3-methyl-*d*-glucose. As will be seen from the experimental part these two γ-glucosides were identical.

To exclude very definitely the possibility of the propyleneoxidic structure for the γ-glucosides, the formation of glucosides of a sugar methylated in position (4) should be studied since in this case the formation of only a pyranose glucoside should be observed if the propyleneoxidic structure is to be excluded for the γ form. Work in this direction is now in progress.

EXPERIMENTAL

Rate of Glucoside Formation—The glucoside formation of glucose and 3-methylglucose was determined in methanol by following the rotation of the solutions. The alcoholic solutions contained 5.0 gm. of dry hydrogen chloride and 0.0344 mol of sugar per liter. Experiments were made at room temperature (23–25°) and at 76°. At the lower temperature, the solutions were placed in 4 dm. polariscope tubes immediately after their preparation and the rotations were measured at various intervals. At 76°, the solutions were mixed in the cold (0°) and small samples were sealed in glass tubes. These tubes were then placed in a bath of boiling carbon tetrachloride (76°) for given intervals of time after which they were quickly cooled to room temperature. The rotations were measured in a 2 dm. tube at 23–25° with sodium D light. They are given in Table I. Specific rotations calculated from these data are plotted in Fig. 1.

TABLE I

Observed Rotations during Glucoside Formation

25°			76°		
Time	Rotation α_D^{24} in 4 dm. tube		Time	Rotation α_D^{24} in 2 dm. tube	
	Glucose	3-Methylglucose		Glucose	3-Methylglucose
hrs.	*degrees*	*degrees*	*hrs.*	*degrees*	*degrees*
0.08	+1.65	+1.79	0.00	+0.88	+0.90
0.67	1.19	1.23	0.08	0.06	−0.54
1.50	0.88	0.79	0.17	0.16	−0.14
2.25	0.63	0.41	0.33	0.31	−0.04
3.00	0.45	+0.12	1.00	0.80	+0.50
5.58	+0.04	−0.54	2.00	1.14	0.89
11.0	−0.18	−0.85	5.00	1.33	1.23
21.0	−0.09	−0.84	10.0	1.39	1.41
27.0	−0.01	−0.81	20.0	1.41	1.54
54.0	+0.19	−0.51	30.0	1.42	1.55
			50.0	+1.41	+1.57

3-Methyl-γ-Methylglucoside—35 gm. of 3-methylglucose were dissolved in 175 cc. of anhydrous methanol containing 0.87 gm. of dry hydrogen chloride. The solution was heated to boiling and was refluxed for exactly 20 minutes. It was then cooled quickly in ice water. During this heating the rotation of the solution changed from the initial value of $[\alpha]_D^{25} = +67°$ to a value of $[\alpha]_D^{25} = -3.2°$. The solution was next treated with silver carbonate to remove the hydrogen chloride, with hydrogen sulfide to remove the excess silver, and was then evaporated to a thick syrup

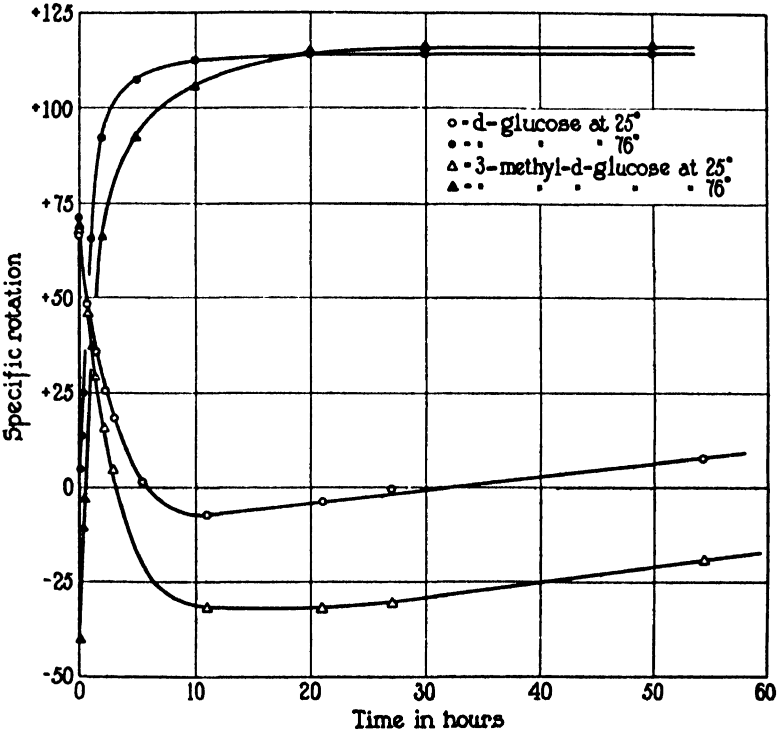

FIG. 1. Glucoside formation

under diminished pressure. The bath temperature during this concentration was kept below 35° to avoid any destruction of the γ-glucoside. The yield of practically colorless syrup was 40 gm. (theoretical 38 gm. for 3-methylmethylglucoside). The syrup was extracted with a total of 900 cc. of dry neutral ethyl acetate leaving a residue of 11.9 gm. The ethyl acetate extract was concentrated at a low temperature to a thick syrup. Weight, 28 gm.

The analysis indicated 6 per cent free sugar, 77 per cent 3-methyl-γ-methylglucoside,[1] and 17 per cent normal 3-methylmethylglucoside (by difference).

Tetramethyl-γ-Methylglucoside—The 3-methyl-γ-methylglucoside was methylated with 280 gm. of methyl iodide and 70 gm. of silver oxide. The methylating mixture was stirred continuously during the process which required 5 hours. The methylated product was extracted with hot chloroform, the extract was dried with anhydrous sodium sulfate, and the chloroform removed under diminished pressure. The product, 27 gm., was remethylated and isolated as before, giving 25 gm. of material. This was distilled at 0.3 to 0.8 mm. giving 23 gm. of product boiling at 95–107°, whose analysis corresponded to 3 per cent free sugar and 79 per cent tetramethyl-γ-methylglucoside. The rotation was

$$[\alpha]_{\mathrm{D}}^{21} = \frac{+0.60^\circ \times 100}{2 \times 4.14} = +7.3^\circ \text{ (in methanol)}$$

20 gm. of the once distilled methylated material were distilled three times at 0.4 to 0.8 mm., the last portion of the distillate being rejected each time. The boiling point of the final product, weight 14 gm., was 95–105°. The analysis indicated 1.3 per cent free sugar and 81 per cent tetramethyl-γ-methylglucoside. The rotation was

$$[\alpha]_{\mathrm{D}}^{23} = \frac{+0.77^\circ \times 100}{2 \times 4.09} = +9.4^\circ \text{ (in methanol)}$$

$$[\alpha]_{\mathrm{D}}^{23} = \frac{+0.72^\circ \times 100}{2 \times 4.07} = +8.8^\circ \text{ (in water)}^{2}$$

and the refractive index was n_{D}^{20}, 1.4426; n_{D}^{25}, 1.4406. It had the following composition.

6.265 mg. substance: 12.109 mg. CO_2 and 4.920 mg. H_2O.
4.121 " " : 19.250 " AgI.
$C_{11}H_{22}O_6$. Calculated. C 52.76, H 8.86, OCH_3 62.03
250.2 Found. " 52.70, " 8.70, " 61.66

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[1] This was determined by hypoiodite titration (Willstätter and Schudel method) for aldose before and after hydrolysis with 0.1 N hydrochloric acid for 10 minutes at 100°.

[2] This final distilled product dissolved in water giving a clear solution. The original undistilled methylated product and even twice distilled samples gave quite opaque water solutions whose rotations could not be easily measured.

The combined residues from the three distillations consisted of 7 per cent free sugar and 80 per cent γ-glucoside. The rotation was

$$[\alpha]_{\mathrm{D}}^{23} = \frac{+1.75° \times 100}{2 \times 4.364} = +20.1° \text{ (in methanol)}$$

and the refractive index was n_{D}^{25}, 1.4440.

Tetramethyl-γ-Glucose—2.52 gm. of the purified tetramethyl-γ-methylglucoside (81 per cent γ form and 18 per cent normal form) were dissolved in 65 cc. of 0.1 N hydrochloric acid, the solution was heated on the steam bath for exactly 10 minutes, and was then cooled in ice water. A hypoiodite titration for aldose indicated 75 per cent hydrolysis of the total glucoside.[3] During the hydrolysis the rotation changed from $[\alpha]_{\mathrm{D}}^{22} = +8.3°$ to $[\alpha]_{\mathrm{D}}^{22} = -8.8°$. This tetramethyl-$\gamma$-glucose was oxidized directly without isolation as indicated below.

2,3,5,6-Tetramethylgluconic Acid-Barium Salt—The solution of tetramethyl-γ-glucose was added to 100 cc. of a solution containing 2.6 gm. of iodine and 5.0 gm. of barium iodide. To this were added 80 cc. of 0.53 N barium hydroxide over a period of 20 minutes during which time the solution was continuously stirred. The mixture was then allowed to stand for 20 minutes after which it was acidified with 5 cc. of 5 N sulfuric acid. The liberated iodine was reduced with sulfur dioxide to iodide ion and the total halide was precipitated with a slight excess of silver sulfate.[4] The combined precipitate was filtered off and the filtrate was treated with hydrogen sulfide. It was then aerated and the silver sulfide removed by filtration. Barium hydroxide was now added until the mixture was slightly alkaline to phenolphthalein, the barium sulfate was centrifuged off and the solution was concentrated under diminished pressure. During this concentration some barium carbonate separated out. This, as well as a small amount of

[3] Hydrolysis of a small sample for 25 hours at 100° with 0.1 N hydrochloric acid gave a solution which by titration indicated 98 per cent hydrolysis to aldose. The rotation was $[\alpha]_{\mathrm{D}}^{22} = +1.2°$.

[4] The silver sulfate was freshly prepared from the calculated quantity of 5 N sulfuric acid by adding silver carbonate until a slight excess of the latter was present. The entire mixture was then added to the solution containing the halide.

barium sulfate not removed by the preceding centrifuging was easily filtered off after the solution had been reduced to 100 cc. volume. The clear solution was further concentrated to a syrup. This was taken up in acetone from which a white amorphous precipitate was thrown out by the careful addition of anhydrous ether. The precipitate was removed, the filtrate reevaporated, and a second crop of material obtained as before. In all, three crops were obtained which weighed 2.5 gm. This material was redissolved in a small amount of water, in which it was exceedingly soluble, acetone was added, and the material was reprecipitated with anhydrous ether. The reprecipitated material contained 7.0 per cent of moisture. Its analysis corresponded to a barium salt of tetramethylgluconic acid. A hypoiodite titration indicated only a trace of aldose.

6.811 mg. substance: 2.561 mg. $BaSO_4$.
4.290 " " : 13.012 " AgI.

$C_{20}H_{38}O_{14}Ba$.	Calculated.	Ba 21.48,	OCH_3 38.80
639.7	Found.	" 22.12,	" 40.22

It had the following rotation.

$$[\alpha]_D^{22} = \frac{+1.23^\circ \times 100}{2 \times 1.852} = +33.2^\circ \text{ (in water)}$$

The yield of barium salt, 2.32 gm. (dry) was 96 per cent of the theory on the basis of 75 per cent hydrolysis of the original tetramethylmethylglucoside.

0.2028 gm. of the barium salt (dry) was dissolved in 3 cc. of water, 6.50 cc. of 0.1 N hydrochloric acid were added, and the solution was made up to exactly 10 cc. This amount of acid is equivalent to the barium present. The rotation of the solution was followed at room temperature (23–25°) for 67 hours, and the equilibrium rotation was then found by warming the solution for short intervals of time. The rotations are given in Table II. The time required to reach half equilibrium rotation is at least 150 hours at 25° which is compatible with the rate of formation of other γ-lactones as indicated by the results of Levene and Simms[5] and of Carter, Haworth, and Robinson.[6] It is entirely different from the rate of formation of the δ-lactones as observed by these investigators.

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[5] Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, **68**, 737 (1926).

[6] Carter, S. R., Haworth, W. N., and Robinson, R. A., *J. Chem. Soc.*, 2125 (1930).

0.5 gm. of the barium salt was dissolved in 12 cc. of water and the calculated equivalent of sulfuric acid (7.53 cc. of 0.19 N) was added. The solution was warmed slightly to coagulate the barium sulfate which was filtered off with the aid of charcoal. The clear filtrate was evaporated to dryness and was heated for a short time at 100° under diminished pressure. The lactone was taken up in 10 cc. of ether and a solution of 0.158 gm. of phenylhydrazine in 2.9 cc. of ether was added. The solvent was evaporated off, as was a second addition of 15 cc. more. To the syrupy residue were added 10 cc. of benzene. From this solvent a flocculent precipitate separated on the addition of ether. The solution was left overnight in the ice box after sufficient ether had been added to bring down a slight precipitate at the boiling point of the mixture. Practically colorless needles separated. After once recrystallizing from benzene they melted at 130.5–132.5° (corrected). A second recrystallization from ether-benzene raised the melting point to 135.3–135.8° (corrected). Haworth, Long, and Plant[7] report a

TABLE II

Lactone Formation at 25°

Time	Rotation α_D^{25} in 2 dm. tube	$[\alpha]_D^{25}$
hrs.	*degrees*	*degrees*
0.0	+0.69	+21.6*
0.3	0.70	21.9
1.0	0.71	22.2
2.0	0.71	22.2
17.5	0.74	23.2
26.0	0.76	23.8
50.0	0.80	25.0
67.0	0.83	26.0
∞	+1.20	+37.6

* The rotation of the free acid is slightly lower than that reported by Drew, Goodyear, and Haworth (Drew, H. D. K., Goodyear, E. H., and Haworth, W. N., *J. Chem. Soc.*, 1237 (1927)) but the difference is not sufficient to affect appreciably the calculation of the rate.

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[7] Haworth, W. N., Long, C. W., and Plant, J. H. G., *J. Chem. Soc.*, 2809 (1927).

melting point of 135–136° for the phenylhydrazide of 2,3,5,6-tetramethylgluconic acid. The melting point of the 2,3,4,6-tetramethyl derivative is 115° and its rotation is $[\alpha]_D = +42.1°$ (in ethanol).

The analysis corresponded to the phenylhydrazide of a tetramethylgluconic acid.

4.444 mg. substance: 9.144 mg. CO_2 and 3.040 mg. H_2O.
4.390 " " : 0.318 cc. N (755 mm. and 25°).
6.206 " " : 17.111 mg. AgI.
$C_{16}H_{26}O_6N$. Calculated. C 56.11, H 7.66, N 8.19, OCH_3 36.27
342.2 Found. " 56.11, " 7.65, " 8.24, " 36.39

The rotation was

$$[\alpha]_D^{21} = \frac{+0.13° \times 100}{2 \times 0.112} = +58° \text{ (in ethanol)}$$

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THE COMPARATIVE RATES OF HYDROLYSIS OF ADENYLIC, GUANYLIC, AND XANTHYLIC ACIDS

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(Received for publication, July 16, 1931)

The ultimate object of the present investigation was the study of the structure of the phosphoric ester of *d*-ribose entering into the structure of ribonucleotides derived from ribonucleic acids. It has been assumed that the ribophosphoric acid derived from inosinic acid differs from that derived from adenylic acid which in turn had been derived from ribonucleic acid. The conclusions to this effect suggested first by Levene and Yamagawa[1] were later corroborated by Embden and Schmidt.[2] They were based on the difference in the rates of hydrolysis of the phosphoric acid residues from the two nucleotides. In order to reach a conclusion as to the details of the differences in the structure of the ribophosphoric acids of the two types it is necessary to be in possession of the free ribophosphoric acid derived from ribonucleic acid.

In a preliminary note recently published by Schmidt[3] the announcement was made that it was possible to prepare the desired ribophosphoric acid by means of a specific enzyme. Our own efforts to prepare ribophosphoric acid from guanylic acid through the action of nucleosidase thus far have been unsuccessful, inasmuch as the enzyme potent for nucleosides is incapable of separating the purine base from nucleotides. In an earlier paper Levene and Weber[4] recorded the fact that their best preparation of nucleosidase was not capable of separating the purine or the pyrimidine bases from the molecule of nucleic acids. It has now been found that the nucleosidases also lack the power to separate the bases from guanylic and adenylic acids.

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[1] Levene, P. A., and Yamagawa, M., *J. Biol. Chem.*, **43**, 323 (1920).

[2] Embden, G., and Schmidt, G., *Z. physiol. Chem.*, **181**, 130 (1929).

[3] Schmidt, G., *Klin. Woch.*, **10**, 165 (1931).

[4] Levene, P. A., and Weber, I., *J. Biol. Chem.*, **60**, 707 (1924).

However, Levene and Jorpes[5] had observed that in nucleotides the rate of acid hydrolysis of both the base and the phosphoric acid residue is modified by small changes in the structure of the base. Thus, in the case of dihydrouridinephosphoric acid, the rate of hydrolysis is of the same order of magnitude as in that of purine nucleotides, whereas the rate of removal of the phosphoric acid residue from uridinephosphoric acid is very low. By analogy, it seemed possible that the rates of hydrolysis of guanylic and xanthylic acids might be different. Indeed, it was found that in a low hydrogen ion concentration, such as exists in a 1 per cent solution of guanylic or of xanthylic acid in water at 50°, the former remains practically unaltered, whereas the latter loses the base to the extent of 45 per cent, leaving the phosphoric acid residue almost intact. Thus a way has been found for the preparation, by chemical means, of *d*-ribosephosphoric acid from guanylic acid.[6]

In the present communication there are discussed three distinct phases of the problem: (1) the preparation of guanylic and xanthylic acids; (2) the effect of nucleosidase on nucleosides and on purine nucleotides; (3) the hydrolysis of guanylic and of xanthylic acids.

EXPERIMENTAL

Preparation of Sodium Guanylate

The amorphous sodium salt suffices for the present purpose. It is prepared by a modification of the method of Steudel and Peiser.[7]

1000 cc. of 5 per cent solution of sodium hydroxide are brought to the boil and in the hot solution 250 gm. of nucleic acid (Boehringer und Sohn) are dissolved. The solution is allowed to stand on a boiling water bath for 8 minutes after which it is freed from a sediment by centrifugalization. The clear solution is neutralized with acetic acid and allowed to stand in the cold overnight. The precipitate thus formed is centrifugalized, washed with a cold 20 per cent solution of sodium acetate, and redissolved by boiling with 500 cc. of a 20 per cent solution of sodium acetate. On cooling, the sodium salt of guanylic acid forms a semigelatinous precipitate. The operation is repeated several times.

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[5] Levene, P. A., and Jorpes, E., *J. Biol. Chem.*, **81**, 575 (1929).

[6] This work is published before the *d*-ribophosphoric acid has been isolated owing to the termination of the visit of Dr. Dmochowski to The Rockefeller Institute.

[7] Steudel, H., and Peiser, E., *Z. physiol. Chem.*, **114**, 201 (1921); **120**, 292 (1922).

The substance prepared in this manner had the following composition.

5.020 mg. substance: 5.321 mg. CO_2 and 2.080 mg. H_2O.
5.240 " " : 0.757 cc. N_2 (27.5°, 755 mm.).
5.430 " " : 27.345 mg. P molybdate.
$C_{10}H_{12}N_5PO_8Na_2$. Calculated. C 29.48, H 2.95, N 17.19, P 7.61
Found. " 28.90, " 3.55, " 16.31, " 7.31

In a 5 per cent solution of sodium hydroxide it has the following rotation.

$$[\alpha]_D^{20} = \frac{-1.72^\circ \times 100}{1 \times 3} = -57.3^\circ$$

On hydrolysis of 2 gm. of the substance with hydrogen chloride in methyl alcohol the yield of guanine chloride was 1.85 gm.

Preparation of Xanthylic Acid

Free xanthylic acid has not hitherto been described although Knopf[8] described its brucine salt. The procedure for its preparation is practically the one which has been employed by Levene and Jacobs for conversion of guanosine into xanthosine and which Knopf used for the preparation of xanthylic acid. 20 gm. of the sodium salt of guanylic acid are dissolved in 20 volumes of water and to the solution 120 cc. of glacial acetic acid are added. Into the solution 300 cc. of a 30 per cent potassium nitrite solution are allowed to flow in a very slow stream and the mixture is stirred mechanically for about 6 hours, at the end of which time the reaction product has lost entirely its sticky viscous character. The reaction product is freed from the remaining traces of guanylic acid by filtration. The xanthylic acid is then precipitated by a 25 per cent solution of neutral lead acetate. The salt is suspended in water and freed from lead by means of hydrogen sulfide. The acid is reconverted into the lead salt. The operation is repeated several times after which the solution of free acid is concentrated under diminished pressure to a smaller volume in a water bath at 35°.

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[8] Knopf, M., *Z. physiol. Chem.*, **92**, 159 (1914).

The acid then settles out in the form of a white powder consisting of microscopic striated balls. By repeating the operation further it will doubtless be possible to obtain the substance in perfectly crystalline form.

The substance had the following composition.

4.810 mg. substance: 5.807 mg. CO_2 and 1.53 mg. H_2O.
0.0896 gm. required 9.93 cc. 0.1 N HCl.
4.495 mg. substance: 26.615 mg. $Mg_2P_2O_7$.

$C_{10}H_{15}N_4PO_9$.	Calculated.	C 32.95, H 4.12, N 15.38, P 8.51
	Found.	" 32.92, " 3.55, " 15.62, " 8.60

The rotation of the substance in 5 per cent sodium hydroxide solution was the following.

$$[\alpha]_{D}^{20} = \frac{-1.25^\circ \times 100}{1 \times 3} = -41.66^\circ$$

Action of Enzymes

The crude enzyme was prepared from pig intestines according to the directions of Levene and Weber.[4] This crude material contained both enzymes, the nucleophosphatase and nucleosidase.

The nucleosidase activity can be seen from Table I.

In Table II is shown the action of the same enzyme on the sodium salt of guanylic acid. It can be seen from this table that when the enzyme is used in a moderate quantity the phosphatase action proceeds at a higher rate than the cleavage of the base. When a large excess of the enzyme is used the two reactions proceed at approximately equal rates.

Efforts were then made either to separate the two enzymes or to find conditions under which the reaction of the phosphatase would be retarded. All possible conditions were not exhausted but thus far whenever the nucleophosphatase action was inhibited, or whenever the nucleophosphatase was removed, the enzyme material failed to remove the base from the nucleotides even when it was active on nucleosides.

In Table III is given the result of the action of an enzyme which

TABLE I

Adenosine

Time	$\frac{1}{50}$ N thiosulfate used	Difference	Reducing ribose	
200 mg. adenosine in 30 cc. H_2O + 20 cc. veronal buffer (pH, 7.0) + 250 mg. enzyme (pig)				
hrs.	*cc.*	*cc.*	*mg.*	*per cent*
0	0.36		0	
4	1.20	0.84	28.0	26.2
22	1.90	1.54	51.2	50.3
50 mg. adenosine in 10 cc. H_2O + 15 cc. phosphate buffer (pH, 7.0) + 150 mg. enzyme (pig)				
0	0.50		0	
2	0.84	0.34	5.7	23.0
22	2.0	1.50	25.0	99.0

TABLE II

Guanylic Acid (Sodium Salt)

Crude Enzyme

Time	$\frac{1}{50}$ N thiosulfate		Reducing ribose		Phosphorus (as $PO_4^{\equiv}$)		
	Found	Increase			Found	Increase	
250 mg. (Na_2-guanylate) + 30 cc. H_2O + 20 cc. veronal buffer (pH, 7.0) + 250 mg. enzyme							
hrs.	*cc.*	*cc.*	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*
0	0.1	0	0		14.44	0	0
4	0.31	0.21	7.0	7.8	15.75	1.3	7.2
22	0.46	0.36	12.0	13.3	22.33	7.9	43.3
250 mg. (Na_2-guanylate) + 30 cc. H_2O + 20 cc. veronal buffer (pH, 7.0) + 1000 mg. enzyme							
0	0.28	0	0		51.6	0	0
4	0.78	0.5	16.7	17.5			
22	2.43	2.15	71.7	78.0	65.0	13.4	73.6

was prepared from the crude enzyme by purifying according to the directions of von Euler and Brunius.[9] This enzyme had a higher nucleophosphatase than nucleosidase potency.

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[9] von Euler, H. and Brunius, E., *Ber. chem. Ges.*, **60**, 1584 (1927).

After dialysis the same enzyme solution used in a large excess apparently removed a larger proportion of base than of phosphoric acid (Table IV). Whether the result was due to the dialysis or to the differences in the proportion of enzyme to substrate cannot as yet be stated. At all events the differences in the rates of action of the two enzymes were not significant.

TABLE III

Adenylic Acid

Enzyme from Pig Kidney Precipitated with Acetone

250 mg. adenylic acid + 30 cc. H_2O + 20 cc. veronal buffer (pH, 7.0) + 250 mg. enzyme.

Time	$\frac{1}{50}$ N thiosulfate		Reducing ribose		Phosphorus (as $PO_4^{\equiv}$)		
	Found	Increase			Found	Increase	
hrs.	*cc.*	*cc.*	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*
0	0.1	0	0	0	13.3	0	
4	0.29	0.19	6.3	6.2	14.75	1.45	6.8
22	0.38	0.28	9.3	9.1	23.62	10.3	48.1

TABLE IV

Guanylic Acid (Sodium Salt)

Enzyme from Pig Kidney Precipitated with Acetone and Dialyzed for 24 Hours

250 mg. (Na_2-guanylate) in 30 cc. H_2O + 20 cc. veronal buffer (pH, 6.0) + 1600 mg. enzyme.

Time	$\frac{1}{50}$ N thiosulfate		Reducing ribose		Phosphorus (as $PO_4^{\equiv}$)	
	Found	Increase			Found	Increase
hrs.	*cc.*	*cc.*	*mg.*	*per cent*	*mg.*	*per cent*
42	2.72	2.72	21.0	98.0	15.5	85.6

No reduction and no phosphorus in dialyzed enzyme.

Purification of the enzyme by aluminium hydroxide and by colloidal iron according to the procedure of Levene and Weber[4] led to enzyme preparations that were very active when tested on nucleosides, but were totally inactive with regard to adenylic and guanylic acids.

TABLE V

Adenylic, Guanylic, and Xanthylic Acids

Time	Adenylic acid, initial pH 3.5				Guanylic acid, initial pH 2.4				Xanthylic acid, initial pH 1.9					
	25°		50°		25°		50°		25°			50°		
	PO_4'''	Sugar	PO_4	Sugar	PO_4'''	Sugar	PO_4	Sugar	α*	PO_4	Sugar	α*	PO_4	Sugar
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*degrees*	*per cent*	*per cent*	*degrees*	*per cent*	*per cent*
0	None	0.0	None	0.0	None	4.8	None	4.8	−1.10	None	0.9	−1.10	None	0.9
23								6.3	−1.10			−0.46		
47		0.2		1.2		4.1		9.0	−1.02		14.9	−0.25		44.5
95	None	1.2	None	5.3	None	4.1	None	13.1	−0.92	None	16.5	−0.12	5.9	47.4

* Measured in a 2 dm. tube with sodium D light.

Hydrolysis of Guanylic and Xanthylic Acids by Hydrogen Ions

Several experiments were made on the hydrolysis of guanylic and xanthylic acids by N hydrochloric acid at temperatures of 50° and 75°. There was no appreciable difference in the rate of hydrolysis of the two substances. However, when aqueous solutions of guanylic (also of adenylic) and of xanthylic acids were hydrolyzed without the addition of mineral acid at 25° and at 50°, then an unmistakable difference was observed in the conduct of the individual substances. Whereas the first two showed but little change under these conditions, xanthylic acid lost 47.4 per cent of its base, without the liberation of any appreciable quantity of phosphoric acid. As yet it cannot be stated whether the differences in behavior were due to differences in the hydrion concentration of the solutions or to the differences in the stabilities of the individual nucleotides.

The results of the observations are given in Table V.

The reducing values were determined by the Hanes[10] modification of the Hagedorn-Jensen method on samples taken directly from the mixtures without treatment. The results thus found were only slightly higher than those on samples which were precipitated first with mercuric acetate, then treated with H_2S, and finally aerated to remove the excess of hydrogen sulfide.

The phosphate determinations were made by precipitating the inorganic phosphate with magnesium ammonium chloride, dissolving this precipitate in dilute hydrochloric acid, and measuring the phosphate by the colorimetric method of Kuttner and Cohen.[11]

Thus a way is given for the preparation of *d*-ribophosphoric acid entering into the structure of guanylic acid derived from ribonucleic acid.

SUMMARY

1. A method for the preparation of xanthylic acid is given.
2. The properties of free xanthylic acid are described.
3. Nucleosidase derived from pig intestinal wall is incapable of removing the base from nucleotides.
4. Xanthylic acid in aqueous solution at 50° loses in 48 hours over 40 per cent of its base without the liberation of free phosphoric acid, thus giving rise to *d*-ribophosphoric acid.

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[10] Hanes, C. S., *Biochem. J.*, **23**, 99 (1929). Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, **135**, 46 (1923).

[11] Kuttner, T., and Cohen, H. R., *J. Biol. Chem.*, **75**, 517 (1927).

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CHEMISTRY OF CHROMOPROTEINS

I. ON THE CHROMOPHORIC GROUP OF THE RHODYMENIA PALMATA

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Considerable advances have been made in the chemistry of chromoproteins, particularly through the work of Kylin,[1] Kitasato,[2] and Lemberg.[3] A good review of the literature can be found in the articles of the last two authors and need not be given here in great detail. In this place it suffices to mention that certain evidence was furnished in favor of the view that the prosthetic groups of the two known chromoproteins, phycoerythrin and phycocyanin, are composed of polypyrrole derivatives. The conclusion was based principally on qualitative tests. A substance isolated by Lemberg seemed to have the composition $C_{34}H_{44}O_8N_4$, which suggested a similarity to the blood and bile pigments.

The slow progress of the work on the structure of chromoproteins is due to two causes: one being the inaccessibility of the starting material, the second, the great resistance of the substance to mild treatment and its instability to drastic treatment. The present work aimed first to develop a method by which the starting material could be made more accessible and second, to isolate some characteristic components that would definitely correlate the substance to known polypyrrole derivatives.

Certain progress has been made by us in both directions. The starting material of previous workers was the crystalline protein which was subsequently digested with pepsin hydrochloric acid.

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* Fellow of The Rockefeller Foundation.

[1] Kylin, H., *Z. physiol. Chem.*, **69**, 169 (1910); **76**, 369 (1912).

[2] Kitasato, Z., *Acta Phytochim.*, **2**, 2, 75 (1925).

[3] Lemberg, R., *Ann. Chem.*, **461**, 46 (1928); **477**, 195 (1930).

The extraction of the proteins from the seaweed was never complete and was always very laborious. Yields were poor, even from the best available source, Japanese nori. From seaweed more readily accessible in America the proteins could be extracted in only infinitely small quantities. It was decided to extract the pigments after preliminary digestion of the entire seaweed inasmuch as the digestion product of the chromoproteins is soluble in amyl alcohol and in this manner can be separated from the other digestion products. The process proved quite successful, with the result that from 1 kilo of the air-dried seaweed 7 gm. of the product which Lemberg termed "phykobilin" were obtained. It was realized that this product contained the chromophoric groups of both phycoerythrin and phycocyanin. However, these two groups are structurally closely related and the phycoerythrobilin can be oxidized easily into a substance very similar to phycocyanobilin.

On purification of the phycobilin (digestion product) by neutral solvents (the details are given in the experimental part) a substance was obtained which had a composition similar to that of the substance obtained by Lemberg by more drastic treatment with hot hydrochloric acid. On treatment with methyl alcoholic hydrogen chloride our substance was esterified and a product was obtained which in its composition resembled the phycoerythrobilinic ester of Lemberg and which this author regarded as the protein-free component of the chromoprotein.

Erythrobilin ester (Lemberg)	C 61.6,	H 7.06,	N 8.85,	OCH_3 6.00
" " "				" 10.40
Ester (Levene and Schormüller)	" 61.03,	" 6.66,	" 7.06,	" 10.40
	" 61.32,	" 7.14,	" 7.55	

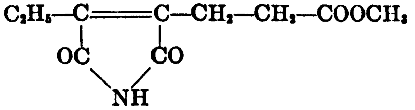
However, our material on hydrolysis with 20 per cent hydrochloric acid for 24 hours showed an amino nitrogen content of 45 per cent of the total nitrogen and hence could not have been the chromophoric component. This fragment may be termed "chromoproteinic acid."

Reduction with Hydriodic Acid

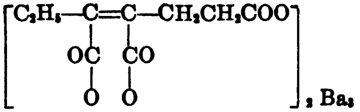
On reduction with hydriodic acid a substance was obtained which had the composition $C_{17}H_{23}NO_4$. It is difficult to state whether even this product was totally free from protein inasmuch as on hydrolysis with strong hydrochloric acid it gave a small quantity of amino nitrogen.

Oxidation with Chromic Acid

The ester on oxidation with chromic acid gave a straw-yellow liquid which, after several redistillations, had approximately the composition $C_{10}H_{13}NO_4$, thus suggesting similarity with an ester of a hematinic acid, more specifically of ethylmaleinimidopropionic methyl ester.

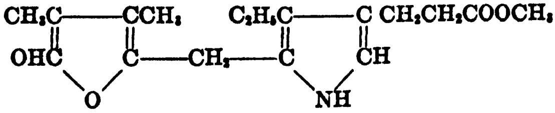


The theoretical values are C 56.87, H 6.16, N 6.6. Found, C 55.91, H 6.25, N 5.83. Inasmuch as a uniform substance could scarcely be expected, the nature of the starting material being borne in mind, the conclusion seemed warranted that the major part of the yellow liquid consisted of the above hematinic acid. This conclusion is further supported by the result of the action of barium hydroxide on this ester. As a result of this action a barium salt of a tribasic acid of the following composition was obtained.



It is significant that basic maleinimide could not be isolated from the product of oxidation.

Upon taking into consideration the latter circumstance and the analytical data on the ester that were obtained on reduction with hydriodic acid, a tentative idea can be formulated as to the composition of the ester; namely, that it is a substance containing a pyrrole and a furan ring in its structure. Thus it may be represented in the following way.



The allocations of two methyl groups and of the hydroxyl group are arbitrary. One ethyl group in place of the two methyl groups would agree equally well with the known facts.

Either of these two substances on oxidation would yield only hematinic acid and none of the basic maleinimides. The furan component on oxidation would be decomposed into volatile acids. The substance would not be readily hydrogenated, and indeed, our efforts to hydrogenate our substance were not successful.

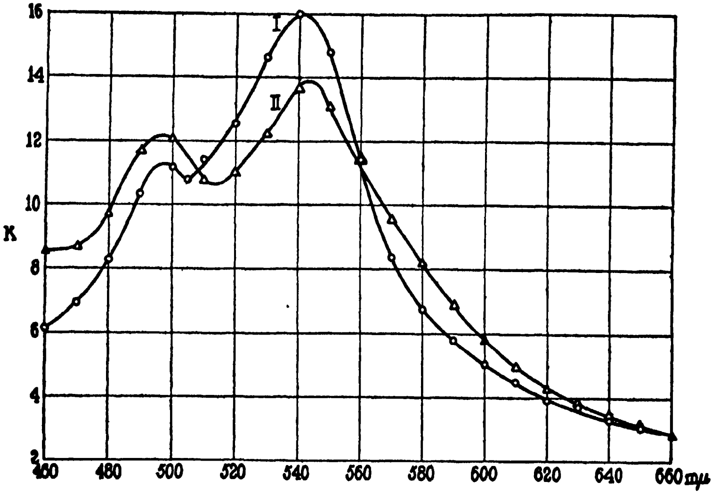


FIG. 1. Absorption curves of chromoproteinic acids.[4] Curve I, chromoproteinic acid obtained from nori. Curve II, chromoproteinic acid obtained from *Rhodymenia palmata*. *K* is the extinction coefficient according to the formula $\frac{I_0}{I} = 10^{cdK}$ where *c* is the concentration expressed in gm. per 100 cc. and *d* the thickness in cm. The measurements were made with a König-Martens spectrophotometer.

We wish to emphasize again that the formula is presented only as a suggestive point of departure for further work and not as a final conclusion as to the structure of the substance.

From the curves in Fig. 1 it may be seen that the chromoproteinic acids obtained from nori and from *Rhodymenia palmata* resemble each other closely.

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[4] We wish to thank Dr. Alexandre Rothen for making the absorption curves.

EXPERIMENTAL

The dried and powdered leaves of *Rhodymenia palmata* (1 kilo) were suspended in 15 liters of water. 150 cc. of concentrated hydrochloric acid and 20 gm. of pepsin dissolved in 500 cc. of ½ per cent hydrochloric acid were added. After standing for 6 to 8 days in the dark, the filtered aqueous solution was extracted three times with amyl alcohol in large separatory funnels. After the first extraction ammonium sulfate was added to the aqueous solution not quite to saturation and the extraction was continued. The combined amyl alcoholic extracts were concentrated under diminished pressure and decanted from the crystallized ammonium sulfate, and the solution further concentrated under reduced pressure with the addition of benzene to remove water as far as practicable.

The oily, dark violet-colored material was filtered from the ammonium sulfate which settled out, and precipitated with a large excess of ether (petroleum ether is not well adapted to this purpose).

The brownish green-colored ether solution was allowed to settle several hours and decanted from the precipitate which was collected on a large Buchner funnel. This material was washed several times with anhydrous ether, dried on the funnel, and immediately placed in a vacuum desiccator over concentrated sulfuric acid. The desiccator was kept under a continuous vacuum for 20 hours and the powdered material was dried for 24 hours in a vacuum over phosphorus pentoxide. In this manner a dark violet-colored, slightly hygroscopic powder was obtained. 1 kilo of algæ yielded about 7 gm. of coloring matter.

For analysis this product was dissolved in amyl alcohol and precipitated with anhydrous ether. This operation was repeated several times. The material was dried at 80°, at 15 mm. The composition of the substance was as follows:

Sample I. 5.185 mg. substance: 10.160 mg. CO_2 and 3.575 mg. H_2O.
8.225 " " : 0.670 cc. N (29°, 760.7 mm.).
Sample II. 4.657 " " : 9.010 mg. CO_2, 2.800 mg. H_2O, and 0.191 mg. ash.

Found.	Fraction I.	C 54.74, H 7.78, N 9.44
"	Ash-free Fraction II.	" 55.75, " 7.10, ash 1.34

In the determination of amino nitrogen a purified sample was previously hydrolyzed for 20 hours with 20 per cent hydrochloric acid.

The total nitrogen and amino nitrogen were also determined on unhydrolyzed material.

Sample I. 7.425 mg. substance:0.634 cc. N (26°, 760 mm.) (Dumas).
17.25 " " :0.48 " " (20°, 761 ") (Van Slyke).
Sample II. 6.620 " " :0.558 " " (20°, 753 ") (Dumas).
20 " " :0.54 " " (19°, 753 ") (Van Slyke).

	Total N *per cent*	Total amino N *per cent*
Unhydrolyzed Fraction I.	9.41	1.60
" " II.	9.72	1.50
Hydrolyzed " II.		5.39

Preparation of Methyl Ester—10 gm. of the dried and finely powdered coloring matter were placed in a dark colored flask with 350 cc. of dry methyl alcohol containing 20 per cent hydrogen chloride and heated for 70 minutes with reflux in a water bath at 80°. The reaction product was thoroughly cooled with ice, diluted with ice water, and extracted with chloroform (five times) until the extract was only slightly colored. The chloroform extracts were washed first with a very dilute solution of sodium acetate (3 to 4 per cent) and then twice with water. The chloroform solution was dried with anhydrous sodium sulfate, concentrated under reduced pressure to a small volume, and precipitated with an excess of petrolic ether (30–40°).

It is essential that all these operations should be carried out with the exclusion of light, since the coloring matters are light-sensitive. This is readily accomplished by painting the outside of all flasks dark red. For analysis the material was dissolved in chloroform and reprecipitated with petrolic ether.

Sample I. 4.951 mg. substance:10.970 mg. CO_2, 2.922 mg. H_2O, 0.050 mg. ash.
5.605 mg. substance: 0.342 cc. N (23.5°, 7.57 mm.).
5.550 " " : 4.410 mg. AgI.
Found (ash-free). C 61.03, H 6.66, N 7.06, OCH_3 10.59, ash 1.00
Sample II. 4.541 mg. substance:10.186 mg. CO_2 and 2.895 mg. H_2O.
8.360 " " : 0.555 cc. N (29°, 760 mm.).
Found (ash-free). C 61.32, H 7.14, N 7.55, ash 0.26

After hydrolysis of the ester with 20 per cent hydrochloric acid the amino nitrogen content was still 3.2 per cent. The ester was therefore treated once more with methyl alcoholic hydrogen chloride for 60 minutes at 80°. However, no important change in the percentage composition of the substance occurred.

The composition of the substance was as follows:

4.340 mg. substance: 9.700 mg. CO_2 and 2.537 mg. H_2O.
Found (ash-free). C 61.80, H 6.63, ash 1.39

Many attempts to obtain the ester in crystalline condition (*e.g.*, from methyl alcohol, dilute ethyl alcohol, chloroform-petroleum ether, benzene-petroleum ether, etc.) were of no avail. Benzene proved to be the most suitable solvent for obtaining the material in a condition which seemed, under the microscope, to be of one definite form but which could not be said to be actually crystalline. The procedure was, however, attended with considerable loss of material.

Reduction of Ester with Hydriodic Acid—To a mixture of 30 cc. of hydriodic acid (sp. gr., 1.96) with 45 cc. of glacial acetic acid contained in a 150 cc. Claisen distilling flask were added 5 gm. of the ester and the solution was heated in a boiling water bath for 20 hours. The excess of free iodine was then removed with the necessary amount of phosphonium iodide, and the entire reaction mixture concentrated to a syrup under diminished pressure. The syrup was dissolved in dilute sodium carbonate and steam-distilled to remove all volatile matter. An oil having a disagreeable odor collected in the receiver.

The distillate was extracted with ether and the ether extract evaporated to dryness yielding an oil which gave a strongly positive reaction with Ehrlich's reagent. Attempts to crystallize the substance or to prepare a picrate were unsuccessful.

The brown-colored solution which remained in the flask after the steam distillation was acidified with dilute hydrochloric acid. A thick brown-colored precipitate was obtained which was filtered on a Buchner funnel and washed with water until free from mineral acid. As the organic acid was only slightly soluble in organic solvents, it was purified by dissolving in sodium carbonate solution and reprecipitating with dilute hydrochloric acid. The precipitate was washed free from hydrochloric acid by repeated centrifuging with water. This material was not entirely ash-free. Its composition was as follows:

4.120 mg. substance: 10.000 mg. CO_2 and 2.695 mg. H_2O.
5.438 " " : 0.240 cc. N (29°, 757 mm.).
Found (ash-free). C 67.87, H 7.49, N 5.08, ash 2.50

Esterification with Methanol of the Product Obtained by Hydriodic Acid Reduction—The acid was covered with a layer of absolute methyl alcohol, saturated with hydrogen chloride, and allowed to stand overnight. The product was then poured on to ice, diluted with water, and extracted with chloroform. The chloroform extract was washed with water, dried with anhydrous sodium sulfate, and concentrated to a small volume under diminished pressure. Precipitation with petrolic ether yielded the ester as a brown fluffy powder. For analysis the ester was purified by dissolving in chloroform, removing all traces of unesterified acid by shaking with dilute sodium carbonate solution, and reprecipitating with petrolic ether. The composition of the substance was as follows:

Sample I. 4.285 mg. substance: 10.080 mg. CO_2 and 2.630 mg. H_2O
5.873 " " : 0.249 cc. N (25°, 745 mm.).
4.977 " " : 3.665 mg. AgI.
Sample II. 6.820 " " : 0.285 cc. N (24°, 750 mm.).
" III. 3.895 " " : 9.415 mg. CO_2, 2.495 mg. H_2O, and 0.065 mg. ash.
10.305 mg. substance: 0.371 cc. N (28°, 761 mm.).

$C_{17}H_{23}O_4N_1$.	Calculated.	C 66.84,	H 7.60,	N 4.60,	OCH_3 10.17	
Found Sample I	Ash-free	" 65.66,	" 6.97,	" 4.76,	" 9.72,	ash 1.70
" II				" 4.82		
" III		" 67.02,	" 7.28,	" 4.12		" 1.66

Oxidation of Ester with Chromic Acid in Glacial Acetic Acid—5 gm. of the ester were dissolved in 150 cc. of glacial acetic acid and to this a solution of 9.5 gm. of chromic acid in 100 cc. of glacial acetic acid were added in one portion. A heavy precipitate settled out at once. The mixture was warmed on a boiling water bath for 1 hour, after which time the precipitate had completely disappeared and the color of the solution turned green. The reaction product was concentrated under diminished pressure to complete dryness. The residue was dissolved in 20 per cent sulfuric acid and extracted eight to ten times with ether. The ether solution was washed with sodium carbonate solution and water and dried with anhydrous sodium sulfate. After removal of the solvent, a small quantity of oxidation product remained. The products of six similar experiments were combined and the oil distilled *in vacuo* (boiling point 180–200°, p = 11 mm.). It was redistilled for analysis. The yield of the 30.0 gm. was about 2.0 gm. The composition of the substance was as follows:

5.370 mg. substance: 11.010 mg. CO_2 and 3.000 mg. H_2O.
10.175 " " : 0.527 cc. N (30°, 760 mm.).
$C_9H_{11}O_4N$. Calculated. C 54.82, H 5.63, N 7.11
$C_{10}H_{13}O_4N$. " " 56.87, " 6.16, " 6.64
Found. " 55.91, " 6.25, " 5.83

A small amount of the distilled oxidation product was placed in a test-tube with a drawn out tip. The oil was dissolved in a small amount of ether. By slightly warming the tube, the ether was driven out and by immersing the tip in 5 per cent pure barium hydroxide solution and cooling the test-tube, it was completely filled to the neck without producing any cloudiness (absence of the formation of barium carbonate). The test-tube was then sealed and shaken overnight. A flocculent precipitate formed which was filtered off. The yield was just enough for an analysis. Determination of the barium identified it as a barium salt of the tribasic hematinic acid.

4.720 mg. substance: 3.975 mg. $BaSO_4$.
$(C_9H_9O_6)_2Ba_3$. Calculated. Ba 49.15. Found. Ba 49.56

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THE ACTION OF ACETIC ANHYDRIDE ON TERTIARY AMINO ACIDS AND DIPEPTIDES. ON CATALYTIC EFFECTS. THE HYDROLYSIS OF ACETYLDIPEPTIDES

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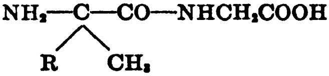
At the outset of this work the preparation of the series of dipeptides of the following type



$$NH_2—\underset{R\quad CH_3}{C}—CO—NHCH_2COOH$$

was contemplated in order that a general study of their properties might be made. Preliminary experiments having indicated that the corresponding bromoacyl-glycines are aminated at an exceedingly low rate and that the isolation of the final products is most difficult, it seemed advisable to have recourse to the "azlactone method of synthesis" which was originated by Mohr[1] and which, in modified forms, has been employed lately with great success by Bergmann and his collaborators.[2] This entailed the preparation of the azlactones of the acetylated tertiary amino acids. While with *d,l*-phenylmethylaminoacetic acid no difficulties were encountered and the azlactone was always obtained in a good yield, the analogous compounds from α-aminoisobutyric acid and *d, l*-isovaline could not be isolated for reasons stated below. The azlactone prepared was converted for identification purposes into N-acetyl-*d*,*l*-phenylmethylaminoacetic acid and its amide. The first of these derivatives had already been obtained, though in not as pure a state, by the action of an excess of acetic anhydride on the amino acid in the presence of pyridine.[3] The pure acetylamino acid melts at 202–203° (corrected).

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[1] Mohr, E., and collaborators, *J. prakt. Chem.*, **81**, 55, 71 (1910); **81**, 473 (1910); **81**, 333, 334 (1910).

[2] Bergmann, M., Stern, F., and Witte, C., *Ann. Chem.*, **449**, 277 (1926). Bergmann, M., and Zervas, L., *Z. physiol. Chem.*, **175**, 154 (1928). Bergmann, M., and Grafe, K., *Z. physiol. Chem.*, **187**, 196 (1930). See also Bergmann, M., Schmidt, V., and Miekeley, A., *Z. physiol. Chem.*, **187**, 264 (1930).

When α-aminoisobutyric acid (0.1 mol) was boiled with an excess of acetic anhydride (100 cc.) deemed sufficient to bring about complete transformation of the amino acid into the azlactone, only solid products, partly insoluble and partly soluble in the excess of acetic anhydride, were obtained (Experiment 1). The total yield of these crude products was very large; since but little amino acid remained to be accounted for, only a small amount of the azlactone formed could possibly have remained and been lost in distillation.[4] The purified solids appear to be complex condensation products. They are probably mixtures of substances of rather high molecular weight. On the other hand, in the absence of such catalysts as traces of chlorine and ammonia commonly present in the commercially available amino acids, and of boiling stones such as were added to the reaction mixtures in the initial experiments, substances of this type are not formed and the reaction takes a fairly normal course (see Table I).

With pure α-aminoisobutyric acid a clear solution was obtained after short boiling. No insoluble materials were formed later, not even when a boiling stone was added to the solution. The main product of this operation, the volatile azlactone, was however lost when the acetic acid and the excess of acetic anhydride were removed by distillation. There remained an oily residue which crystallized spontaneously after removal by means of xylene of the last traces of acetic anhydride and of azlactone. The crystals (yield, 20 per cent of the theory) were those of a compound $C_{10}H_{16}O_3N_2$ (Experiment 3). For a summary of these experiments, see Table I.

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[3] Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **79**, 102 (1928).

[4] Some time later, a short passage in Mohr, E., and Geis, T., *J. prakt. Chem.*, **81**, 56 (1910), referring specifically to the matter, came to our attention. Mohr states that in an experiment with 2 mols of acetic anhydride probably little azlactone was lost and that the bulk of the α-aminoisobutyric acid remained in the residue from the distillation in the form of an unidentified material.

An experiment similar to the foregoing was then carried out with pure anhydrous *d,l*-isovaline (Experiment 1). The course of the reaction, however, was as far as could be seen, not modified by the addition of a small amount of ammonium chloride and of a boiling stone to the mixture of this amino acid with acetic anhydride (Experiment 2).

Observations made at a later time in the course of this work may be mentioned here. The dipeptide α-aminoisobutyryl-α-aminoisobutyric acid undergoes rather readily dehydration in a medium of hot glacial acetic acid. The rate of this reaction seems to be largely determined by the amount of impurities, that is, of traces of ammonia, contained in the dipeptide used. A sample of a high degree of purity (no coloration and no cloudiness with Nessler's reagent) gave a clear solution with this solvent, whereas a slightly less pure material (which showed but a slight cloudiness but no coloration on long standing with Nessler's reagent) gave,

TABLE I

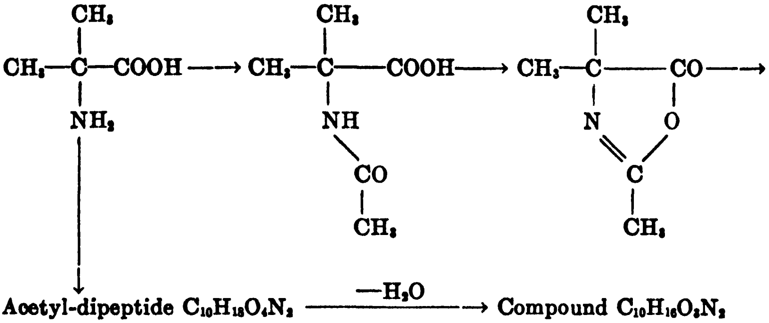
Action of Acetic Anhydride on α-Aminoisobutyric Acid

Weights of the solid products obtained in Experiments 1, 2, and 3 with 10.4 gm. (0.1 mol) of α-aminoisobutyric acid and 100 cc. of 95 per cent acetic anhydride.

Experiment No.	Nature of amino acid used	Boiling stone added	In acetic anhydride		Nature of Residue B
			Insoluble fractions (Residue A) washed	Soluble products, crude (Residue B)	
1	Kahlbaum's. Contained traces of Cl and NH_3	At very start	0.65 gm. (No. 284)	7.25 gm. (Residue B_1)	Complex mixture
2	Purified, free of traces of Cl and NH_3	At very start	0.39 gm. (No. 287)	2.85 gm. (Residue B_2)	Lactone $C_{10}H_{16}O_3N_2$ and another material
3	Same as for Experiment 2	30 min. after start	0.00 gm.	2.08 gm.	Lactone $C_{10}H_{16}O_3N_2$

when dissolved in hot acetic acid, a strongly opalescent solution. In both cases there was α-aminoisobutyric acid anhydride formed. Because of lack of material, quantitative experiments under conditions strictly comparable could not be carried out.

The steps leading to the formation of the compound $C_{10}H_{16}O_3N_2$ from α-aminoisobutyric acid are the following.


CH_3—C(CH_3)(NH_2)—COOH
CH_3—C(CH_3)(NH—CO—CH_3)—COOH
CH_3—C(CH_3)—CO, N=C(CH_3)—O
Acetyl-dipeptide $C_{10}H_{18}O_4N_2$
$-H_2O$
Compound $C_{10}H_{16}O_3N_2$

A certain amount of azlactone of N-acetyl-α-aminoisobutyric acid formed reacts with an equivalent quantity of unchanged amino acid (acetylation takes place slowly[5]) to give an acetyl-dipeptide (broad sense designation) which by loss of water in one of various possible ways is then converted into the final product.

In Table II are given the structural formulæ of the cyclic compounds $C_{10}H_{16}O_3N_2$ that can be derived from N-acetyl-α-aminoisobutyryl-α-aminoisobutyric acid. Inasmuch as the isolated substance behaves on titration as would a typical lactone, but one of the first three of these structures comes into consideration for this compound. The first (and the only one hitherto known) lactone of an N-acyl-dipeptide has been prepared by Mohr and Stroschein.[6] N-Benzoyl-*d,l*-alanyl-α-aminoisobutyric acid when heated with acetic anhydride to 100° underwent dehydration. Mohr ascribed to his substance a structure similar to that of Compound I (see Table II) but no evidence to that effect was produced. Its behavior towards water was not investigated.

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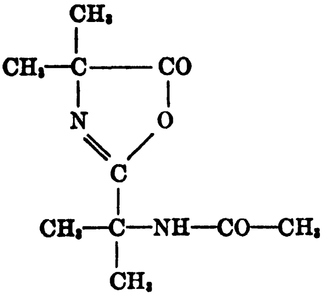
[5] Quite some time elapses before even pure α-aminoisobutyric acid (0.1 mol) is completely dissolved in the chosen amount of acetic anhydride (100 cc.). *d,l*-Isovaline and *d,l*-phenylmethylaminoacetic acid are dissolved much more rapidly under identical conditions.

[6] Mohr, E., and Stroschein, F., *J. prakt. Chem.*, 81, 478, 499 (1910).

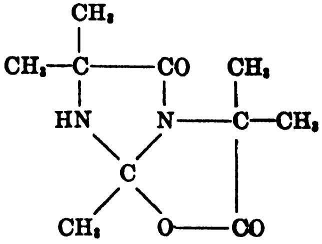
The lactone $C_{10}H_{16}O_3N_2$ is hydrolyzed by water at room temperature and gives almost quantitatively N-acetyl-α-aminoisobutyryl-α-aminoisobutyric acid. This substance was also prepared in a different way, that is, by acetylation of the corresponding

TABLE II

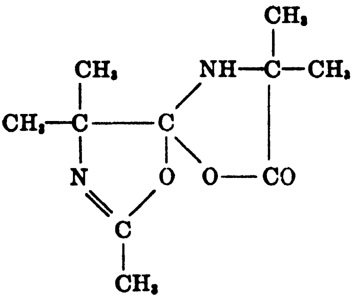
Compounds $C_{10}H_{16}O_3N_2$



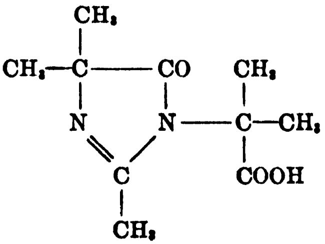
I



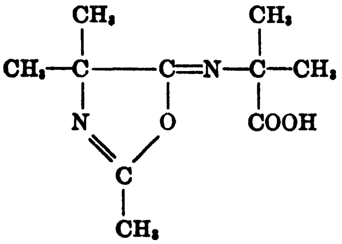
II



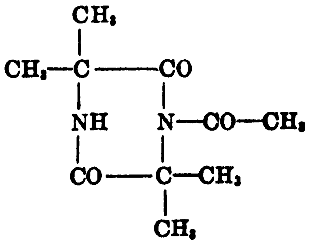
III



IV

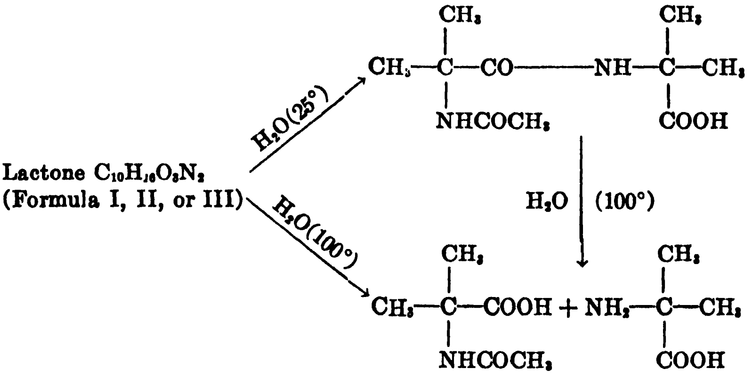


V



VI

dipeptide under conditions described in the experimental part. The lactone is decomposed by water at 100°. The solution contains an equimolecular mixture of α-aminoisobutyric acid and of N-acetyl-α-aminoisobutyric acid. These substances were actually isolated. The nature of the latter was established by a direct comparison with the compound obtained on acetylation of α-aminoisobutyric acid with 1.1 mols of acetic anhydride in acetic acid solution. This substance had not yet been prepared.


Lactone $C_{10}H_{16}O_3N_2$
(Formula I, II, or III)
H_2O(25°)
H_2O(100°)
CH_3—C(CH_3)($NHCOCH_3$)—CO—NH—C(CH_3)(COOH)—CH_3
H_2O (100°)
CH_3—C(CH_3)($NHCOCH_3$)—COOH + NH_2—C(CH_3)(COOH)—CH_3

The very formation of these scission products and the ease with which this reaction takes place are of distinct significance. But how premature any speculation at this stage of the work as to the probable structure of the lactone $C_{10}H_{16}O_3N_2$ would have been is shown by the following. N-Acetyl-α-aminoisobutyryl-α-aminoisobutyric acid was not only found to be as unstable towards boiling water as the lactone itself but also to give the same products of scission. This last finding was wholly unexpected. It is known that the acetyl derivatives of dipeptides when boiled for a moderate length of time with dilute mineral acids undergo deacetylation and yield the acid salts of the dipeptides. Also it had been previously established that dipeptides, such as glycyl-α-aminoisobutyric acid, containing a tertiary amino acid as second component, were, unlike the peptides wholly composed of ordinary amino acids, very resistant towards hydrolytic agents (erepsin and alkali).[7]

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[7] Levene, P. A., Steiger, R. E., and Bass, L. W., *J. Biol. Chem.*, **82**, 156, 164 (1929). Levene, P. A., Bass, L. W., and Steiger, R. E., *J. Biol. Chem.*, **82**, 168, 169 (1929).

α-Aminoisobutyryl-α-aminoisobutyric acid was then prepared. It was found to be absolutely resistant to the action of boiling water (4 hours). Thus, with the acetyl derivative of this dipeptide, a sharply marked case of substitutional activation has been revealed.[8] As is readily surmised, the nature of the two amino acid components of the system determines the instability of the peptidic linkage, the very degree of this instability depending largely upon the structure of the first amino acid. This was established in the following way. N-Acetyl-glycyl-α-aminoisobutyric acid was prepared. This is also hydrolyzed by boiling water but only to the extent of 10 per cent in the course of 4 hours (against 90 per cent as found in the case of the former compound). Whether deacetylation takes place or whether the peptide linkage is severed was not determined. N-Acetyl-*d*,*l*-α-aminophenylmethylacetyl-α-aminoisobutyric acid was then prepared by condensation of the azlactone of N-acetyl-*d*,*l*-phenylmethylaminoacetic acid with α-aminoisobutyric acid. This acetyl-dipeptide could not be obtained by the method seemingly general for reactions of this kind proposed by Mohr and Stroschein[9] and adopted by Bergmann and his collaborators[2]; namely, by condensing the components in alkaline acetone-water mixtures. The azlactone was invariably hydrolyzed to the corresponding acetyl-amino acid and no other reaction took place. The acetyl-dipeptide was, however, very readily formed when the azlactone was added to a hot suspension of the amino acid (used in slight excess) in glacial acetic acid. This procedure is similar to Knoop and Blanco's method[10] of acetylating amino acids by means of acetic anhydride. It is believed that it will prove valuable not only in the case of tertiary amino acids but also in the more general instance of most other amino acids as well as in the case of peptides.

This last acetyl-dipeptide is even more readily decomposed by boiling water then the one discussed first. Hydrolysis to the extent of 94 per cent was observed in the course of 4 hours. The products of scission were isolated. The reaction is as follows:

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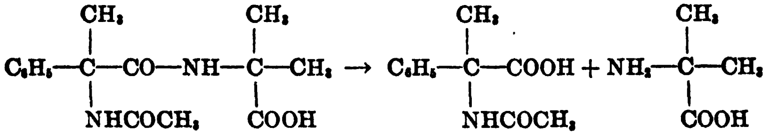
[8] So far as could be ascertained, no record is to be found in the literature of any attempt to hydrolyze acetyl-dipeptides at the pH and temperature of their boiling aqueous solutions. The extent of the hydrolysis increases definitely with the concentration of the solution.

[9] Mohr, E., and Stroschein, F., *J. prakt. Chem.*, **82**, 334 (1910).

[10] Knoop, F., and Blanco, J. G., *Z. physiol. Chem.*, **146**, 273 (1925).



$$C_6H_5-\underset{NHCOCH_3}{\overset{CH_3}{C}}-CO-NH-\underset{COOH}{\overset{CH_3}{C}}-CH_3 \rightarrow C_6H_5-\underset{NHCOCH_3}{\overset{CH_3}{C}}-COOH + NH_2-\underset{COOH}{\overset{CH_3}{C}}-CH_3$$

These findings on the instability of substituted dipeptides containing a tertiary amino acid as first component afford a ready interpretation of certain observations made by Mohr and Geis[11] as long ago as 1910. These authors had prepared N-benzoyl-α-aminoisobutyryl-glycine by fusing the azlactone of N-benzoyl-α-aminoisobutyric acid with glycine. The reaction product was recrystallized several times from boiling water. It yielded a mixture of crystals which softened at 175° before melting at 191° and were soluble in about 13.8 parts of boiling water. The molecular weight determined by titration was only 254.4 instead of 264.15. This material undoubtedly contained about 17 per cent of N-benzoyl-α-aminoisobutyric acid (this softens at 195°, melts at 198°, dissolves in 28 parts of boiling water, and has a molecular weight of 207.11) formed by partial decomposition of the original substance in boiling water.

EXPERIMENTAL

Action of Acetic Anhydride on α-Aminoisobutyric Acid

Experiment 1—100 cc. of 95 per cent acetic anhydride (about 0.5 mol) were added to 10.4 gm. (0.1 mol) of finely powdered α-aminoisobutyric acid (Kahlbaum's) containing traces of ammonium chloride. A boiling stone was added. The 200 cc. Pyrex round bottom flask was equipped with a reflux condenser and immersed in an oil bath kept at 150°. Boiling took place after 8 minutes. No clear solution was obtained at any time. The suspension was kept at boiling temperature for ½ hour. It was then rapidly cooled down to room temperature. It contained an appreciable amount of insoluble material. This was filtered off with suction. It was thoroughly washed with xylene (Residue A_1).

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[11] Mohr, E., and Geis, T., *J. prakt. Chem.*, 81, 71 (1910).

Residue A_1 was well washed with water and then with absolute alcohol. It weighed dry, 0.65 gm. When heated to 250° this material did not melt.

No. 284.

4.610 mg. substance: 9.360 mg. CO_2 and 3.205 mg. H_2O.
5.845 " " : 0.749 cc. N (27°, 762.5 mm.) (Dumas).
5.125 " " : 10.395 mg. CO_2 and 3.700 mg. H_2O.
6.360 " " : 0.800 cc. N (23.5°, 764.7 mm.) (Dumas).
Found. C 55.36, 55.31; H 7.77, 8.07; N 14.62, 14.53

The filtrate of Residue A_1 was concentrated under reduced pressure. There remained an oily red residue. It was dissolved in xylene, which was then evaporated off. This operation was repeated several times in order to remove any remaining acetic anhydride and some other volatile material. The temperature of the bath was finally raised to 90°. The flask with its contents was then kept in a vacuum desiccator over phosphorus pentoxide, soda-lime, and paraffin chips. The residue became solid after some time. It weighed 7.25 gm. (Residue B_1). It was readily soluble in hot benzene but no deposit was formed on cooling nor was a crystalline precipitate obtained when other solvents were added to this solution. The remainder of Residue B_1 was boiled for several hours with water. The filtrate containing some water-soluble substances was discarded. This product was dissolved in boiling absolute alcohol; the solution was clarified with norit and concentrated to dryness under reduced pressure. The residue was suspended in water, filtered off, washed with water, and dried. When heated to 250° it did not melt. It was soluble in 0.1 N NaOH; insoluble, however, in 0.1 N HCl.

No. 302.

4.800 mg. substance: 9.690 mg. CO_2 and 3.485 mg. H_2O.
6.965 " " : 0.879 cc. N (24°, 769.2 mm.) (Dumas).
Found. C 55.05, H 8.12, N 14.38 (Dumas)
" 0 (Van Slyke)
0.1600 gm. substance: 2.50 cc. 0.1 N NaOH (titration, phenolphthalein).
Acidity of 100 gm., in cc. of 1.0 N NaOH, 156.3.

A small amount of a material (No. 303) identical with this one was obtained in very much the same manner from the reaction product (Residue B_2) of the following experiment.

Experiment 2—The reaction was carried out in the same way as in Experiment 1. However, pure α-aminoisobutyric acid, free of traces of chlorine and ammonia, was used. A boiling stone consisting of a fragment of a porous porcelain plate was added to the mixture from the start. No clear solution was obtained at any time. The insoluble material was separated by filtration from the supernatant liquid. It was washed with xylene (Residue A_2).

Residue A_2 was thoroughly washed with water and then with absolute alcohol. When dry, it weighed 0.39 gm. Its composition was then that of a compound $C_{17}H_{30}O_5N_4$ or $C_{34}H_{60}O_{10}N_8$ (C 55.10, H 8.16, N 15.14). However, whether this material was pure or was but a mixture of substances could not be determined. When heated to 250°, it did not melt. It was slightly, but entirely, soluble in pyridine.

No. 287.
5.200 mg. substance: 10.500 mg. CO_2 and 3.815 mg. H_2O.
6.790 " " : 0.916 cc. N (30°, 755.3 mm.) (Dumas).
Found. C 55.06, H 8.20, N 15.09

The filtrate of Residue A_2 was concentrated under reduced pressure. The acetic anhydride remaining in the residue was removed by means of xylene in the manner indicated before. The product of the reaction crystallized in the course of the last evaporation. The bath was then at 70°. Some oil (the azlactone of N-acetyl-α-aminoisobutyric acid) was given off when the temperature of the bath was raised to 85–90°. The crystalline residue was dried in a vacuum desiccator over phosphorus pentoxide, soda-lime, and paraffin chips. It weighed 2.85 gm. (Residue B_2). This product was dissolved in hot benzene. The crystals deposited on cooling were in the main those of a lactone $C_{10}H_{16}O_3N_2$ but their separation from those of an accompanying impurity of more complex nature could not be accomplished by fractional crystallization. The mixture was therefore treated with water at 20–25°, wherein the lactone is readily soluble. There remained an insoluble fraction which was filtered off (the filtrate yielded, on slow concentration, crystals of N-acetyl-α-aminoisobutyryl-α-aminoisobutyric acid (see that paragraph)). It was thoroughly washed with water and dried. When heated to 250°, it did not melt.

No. 303.

4.020 mg. substance: 8.080 mg. CO_2 and 3.025 mg. H_2O.
6.360 " " : 0.793 cc. N (25.5°, 758.3 mm.) (Dumas).
Found. C 54.81, H 8.42, N 14.21
0.1390 gm. substance: 2.16 cc. 0.1 N NaOH (titration, phenolphthalein).
Acidity of 100 gm., in cc. of 1.0 N NaOH, 155.4.

Experiment 3. Preparation of Lactone $C_{10}H_{16}O_3N_2$—100 cc. of 95 per cent acetic anhydride (about 0.5 mol) were added to 10.4 gm. (0.1 mol) of finely powdered pure α-aminoisobutyric acid, free of traces of chlorine and ammonia. (This amino acid was not in any way different from that employed in Experiment 2. Both samples were taken from the same batch.) The 200 cc. round bottom Pyrex flask was equipped with a reflux condenser and was immersed in an oil bath kept at 150°. The mixture started to boil after 6 minutes. After 4 minutes of effective boiling all the amino acid had dissolved. A clear solution was obtained. Only some time after this was a boiling stone added to the mixture. The solution was kept at boiling temperature for a total of 30 minutes. As it remained perfectly clear during all this time and even after cooling to room temperature, it was directly concentrated under reduced pressure. The oily residue was repeatedly taken up in xylene, this being evaporated off as often in between times. The acetic anhydride and some azlactone of N-acetyl-α-aminoisobutyric acid thus being removed, the residue crystallized spontaneously in the course of the last evaporation. The flask with its contents was kept in a vacuum desiccator over phosphorus pentoxide, soda-lime, and paraffin chips. The crystals weighed 2.08 gm. They were those of a lactone $C_{10}H_{16}O_3N_2$.

Lactone $C_{10}H_{16}O_3N_2$

It can be easily prepared by the action of acetic anhydride on pure α-aminoisobutyric acid. The method of preparation is given above under Experiment 3; the conditions must be rigorously observed. The crude material obtained (yield, 20 per cent of the theory) was recrystallized from hot benzene. The crystals obtained were constantly kept stored over phosphorus pentoxide. They melted at 143–144° (corrected) with slight preliminary sintering. For analysis the substance was dried at 100° under reduced pressure. It then sublimed in part, but no alteration took place.

No. 311.
4.845 mg. substance: 10.045 mg. CO_2 and 3.064 mg. H_2O.
6.087 " " : 6.96 cc. N (24.5°, 755.6 mm.) (Dumas).
$C_{10}H_{16}O_3N_2$ (212.15). Calculated. C 56.56, H 7.60, N 13.21
Found. " 56.53, " 7.07, " 13.05

A freshly prepared solution of the lactone in water (in which the substance is readily soluble) contained no amino nitrogen nor was any detectable at any time on keeping. Solutions of the lactone in 2 mols of 0.1 N HCl and of 0.1 N NaOH were kept for 24 hours at 25° and amino nitrogen determinations were made. As in the case with pure water, no amino nitrogen was found. The lactone is hydrolyzed by water at room temperature to N-acetyl-α-aminoisobutyryl-α-aminoisobutyric acid and decomposed by water at 100° to N-acetyl-α-aminoisobutyric acid and α-aminoisobutyric acid. The lactone seems to be stable in absolute alcohol at room temperature.

Hydrolysis of Lactone $C_{10}H_{16}O_3N_2$ with Water at 25°

The lactone $C_{10}H_{16}O_3N_2$ is hydrolyzed by water at room temperature. Ring opening takes place; ultimately N-acetyl-α-aminoisobutyryl-α-aminoisobutyric acid (see that paragraph) is formed. Little can be said about the rate of formation of this derivative. A freshly prepared solution of the lactone in water is distinctly acid towards acid indicators. When 0.1 N NaOH is added gradually to such a solution, phenolphthalein being used as an indicator, the alkali is very rapidly consumed up to a certain stage in the experiment, after which the pink coloration is more persistent. When the end-point is practically reached, it will only disappear in the course of several hours. A permanent coloration was attained with the following.

0.1061 gm. (0.0005 mol) lactone: 5.01 cc. 0.1 N NaOH.
Calculated for 0.0005 mol R·COOH: 5.00 cc. 0.1 N NaOH.

No amino nitrogen was detected in this solution.

Hydrolysis of Lactone $C_{10}H_{16}O_3N_2$ with Water at 100°, and Isolation of the Products of Hydrolysis

About 80 cc. of boiling water were added to 0.4243 gm. (0.002 mol) of the pure lactone $C_{10}H_{16}O_3N_2$ (No. 311) contained in a 100 cc. volumetric flask. The clear solution was kept at boiling temperature for 4 hours. Water was then added to bring its volume to 100 cc. No attention was paid to a slight cloudiness formed and the following determinations were made on this solution (No. 312).

4.00 cc. gave 1.74 cc. N at 21° and 761 mm. (Van Slyke, 45 minutes).[12]
10.00 " required 4.20 cc. 0.1 N HCl (Kjeldahl).
10.00 " consumed 2.01 " 0.1 " NaOH (titration, phenolphthalein).

These results are to be interpreted as follows:

Calculated for a mixture of 0.002 mol of α-aminoisobutyric acid and 0.002 mol of N-acetyl-α-aminoisobutyric acid in 100 cc. of solution

$\frac{NH_2\text{ nitrogen}}{\text{Total nitrogen}} = 0.50$. Acidity (on 100 cc.) = 20.00 cc. 0.1 N NaOH[13]

Found

$\frac{NH_2\text{ nitrogen}}{\text{Total nitrogen}} = 0.40$. Titration (on 100 cc.) = 20.10 cc. 0.1 N NaOH

The solution which remained after these determinations was clarified by filtration and then evaporated to dryness under reduced pressure. The crystalline residue was kept in a vacuum desiccator over phosphorus pentoxide until completely dry. It was then rapidly extracted with a small amount of absolute alcohol.

The insoluble fraction was washed with absolute alcohol (the washings were discarded) and dried. This material (No. 318) was found to contain 13.71 per cent of total nitrogen (Kjeldahl) and 13.33 per cent of amino nitrogen (Van Slyke). A further and larger amount of the same material had been isolated previously from the residue of another hydrolysis experiment similar to the foregoing. This residue was dissolved in a hot mixture of water and alcohol. The clear solution on cooling in ice and water yielded a microcrystalline precipitate which was insoluble in cold alcohol. It was dried at 90°. The analysis showed this to be *α-aminoisobutyric acid.*

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[12] The rate of deamination of tertiary aliphatic amino acids is considerably lower than that of ordinary amino acids. Correct amino nitrogen figures, with Van Slyke's apparatus, are obtained only after 45 minutes of shaking. See Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **76**, 302, 318 (1928).

[13] α-Aminoisobutyric acid does not consume any alkali, phenolphthalein being used as an indicator.

No. 288.
5.880 mg. substance: 10.080 mg. CO_2 and 4.560 mg. H_2O.
5.195 " " : 0.606 cc. N (23°, 759.7 mm.) (Dumas).
10.000 " " : 2.37 " " (23°, 762.1 " (Van Slyke).[12]
$C_4H_9O_2N$ (103.08). Calculated. C 46.57, H 8.80, N 13.59
Found. " 46.74, " 8.67, " 13.44 (Dumas)
" 13.33 (Van Slyke)

The alcoholic extract (see above) was evaporated to dryness. The residue was completely dried over phosphorus pentoxide and was dissolved in boiling ethyl acetate. The crystals deposited on cooling melted at 192–193° (uncorrected) and at 194–195° (corrected). The substance thus isolated was *N-acetyl-α-aminoisobutyric acid* for a mixture with the somewhat purer substance obtained on direct acetylation of α-aminoisobutyric acid (see the following paragraph) melted at the same temperature.

N-Acetyl-α-Aminoisobutyric Acid

7.2 gm. of 95 per cent acetic anhydride (0.066 mol) were added to a warm mixture of 6.2 gm. (0.06 mol) of α-aminoisobutyric acid and 60 gm. of glacial acetic acid.[14] This mixture was boiled for a short time; a clear solution resulted. It was allowed to cool. It was then concentrated to a small volume under reduced pressure. The acetyl derivative crystallized. Toluene was added to the mixture and then was evaporated off, this in order to remove all the acetic acid present. The dry residue was suspended in a mixture of toluene and heptane (b.p. 90–100°). It was filtered off and washed with heptane. The air-dried material weighed 9.30

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[14] The general method devised by Knoop and Blanco (Knoop, F., and Blanco, J. G., *Z. physiol. Chem.*, **146**, 273 (1925)) was used.

gm. (theory, 8.71 gm.). It contained some unchanged amino acid. The complete separation was effected as follows: The crude material was extracted with hot absolute alcohol. The insoluble fraction was discarded and the filtrate was concentrated to dryness. This residue was almost entirely soluble in hot absolute alcohol. The solution was clarified with norit and concentrated to a small volume under reduced pressure. Crystallization took place. The crystals were washed with a small amount of cold absolute alcohol and then more freely with ethyl acetate. They were dissolved in boiling ethyl acetate. An insoluble fraction was removed by filtration and discarded. Abundant crystallization took place on cooling. The analysis of this substance was as follows:

No. 319.
4.970 mg. substance: 9.085 mg. CO_2 and 3.260 mg. H_2O.
5.198 " " : 0.437 cc. N (25°, 754.5 mm.) (Dumas).
$C_6H_{11}O_3N$ (145.10). Calculated. C 49.62, H 7.64, N 9.66
Found. " 49.99, " 7.34, " 9.56 (Dumas)
" 0 (Van Slyke)
0.1451 gm. substance: 9.99 cc. 0.1 N NaOH (titration, phenolphthalein).
Calculated for 0.001 mol: 10.00 cc. 0.1 N NaOH.

This compound is quite soluble in water and in alcohol, but much less so in hot ethyl acetate. It melted at 193–194° (uncorrected) and at 195–196° (corrected) with preliminary sintering.

α-Aminoisobutyryl-α-Aminoisobutyric Acid

This dipeptide has already been obtained by amination for 3 days of α-bromoisobutyryl-α-aminoisobutyric acid[15] with 5 parts of 25 per cent ammonium hydroxide at 37°. A modification of this procedure and some information regarding the stability of this dipeptide are given in the following. A mixture of 1 part of crude bromo compound and 10 parts by weight of ammonium hydroxide of sp. gr. 0.90 was kept for 10 days at 41° ± 2°. The dry residue of this solution still contained a large amount of untransformed bromo compound in the form, however, of the stable ammonium salt. It is best treated in the cold with a slight excess of dilute hydrochloric acid which decomposes this salt with precipitation of the free acid and dissolves the dipeptide. The filtrate containing the latter was then freed of halogen ions in the usual way and concentrated. The dipeptide was precipitated with alcohol. Yield, over 24 per cent (rather large losses of material were sustained before the method by extraction with acid was adopted). The product obtained contained traces of ammonia. It was recrystallized twice from water and alcohol. The final product gave no coloration with Nessler's reagent nor did the mixture become cloudy on long standing. It dissolved in hot glacial acetic acid to give a clear solution. The analysis was as follows:

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[15] Abderhalden, E., and Gebelein, F., *Z. physiol. Chem.*, **152**, 129 (1926).

No. 349.
4.539 mg. substance: 8.520 mg. CO_2 and 3.419 mg. H_2O.
4.340 " " : 0.557 cc. N (27°, 761 mm.) (Dumas).
$C_8H_{16}O_3N_2$ (188.15). Calculated. C 51.02, H 8.57, N 14.89
Found. " 51.18, " 8.42, " 14.61
Moisture, 5.69 per cent

The mother liquors of crystallization of the preceding were combined. They yielded a less pure material (Sample 2). It gave no coloration with Nessler's reagent but the solution became cloudy on long standing. Its solution in hot glacial acetic acid was strongly opalescent.

This dipeptide is not hydrolyzed by boiling water. A solution of 0.1822 gm. of dipeptide in about 20 cc. of water was boiled for 4 hours under a reflux. Its volume was then brought to 25 cc. The following determinations were made on this solution (No. 351).

2.00 cc. gave 1.95 cc. N at 23° and 752 mm. (Van Slyke, 45 minutes).[12]
10.00 " required 7.89 cc. 0.1 N HCl (Kjeldahl).

Hence the amino nitrogen:total nitrogen ratio found was 0.49 against 0.50 as calculated and 0.51 as found for the blank.

α-Aminoisobutyric Acid Anhydride

This compound is well known. It has been obtained by many authors in several various ways and more recently again by Abderhalden and Gebelein[15] in the following manner. α-Aminoisobutyryl-α-aminoisobutyric acid was heated with diphenylamine until dehydration which set in at 180° was complete (200°). The observation has now been made that this dipeptide is rather readily converted into its anhydride by hot glacial acetic acid and hence is always obtained in more or less large amounts as a by-product when acetylation of the dipeptide by means of acetic anhydride (used in small excess) is carried out in that solvent. The rate of dehydration seems to be largely determined by the amount of traces of ammonia present in the starting material used. An acetylation experiment performed on a highly pure sample (No. 349) is described in one of the following paragraphs. The yield of anhydride was low and that of acetyl-dipeptide good. A summary of a similar experiment with a slightly less pure material is given here. 1.88 gm. of moist dipeptide (Sample 2; 0.0094 mol, dry) gave with 10 cc. of glacial acetic acid, after some heating and shaking, a strongly opalescent solution. It deposited crystals of anhydride on cooling before the acetic anhydride could be and was added. The suspension was again brought to its boiling point and then was allowed to cool. It was diluted with water and evaporated to dryness. The residue was treated with sodium hydroxide until alkaline towards phenolphthalein. The anhydride was filtered off, washed with water, then with dilute acid, and again with water. It weighed dry 0.668 gm. (yield, 42 per cent of the theory). The filtrate and washings were discarded. This material was recrystallized from boiling acetic acid. The crystals were washed with acetic acid and with absolute alcohol. They weighed 0.44 gm.

No. 358.
4.020 mg. substance: 8.380 mg. CO_2 and 2.950 mg. H_2O.
4.268 " " : 0.618 cc. N (27°, 758 mm.) (Dumas).
$C_8H_{14}O_2N_2$ (170.13). Calculated. C 56.43, H 8.29, N 16.47
Found. " 56.84, " 8.21, " 16.43

N-Acetyl-α-Aminoisobutyryl-α-Aminoisobutyric Acid

1. Preparation from the Lactone $C_{10}H_{16}O_3N_2$—This substance can be easily prepared in a pure state. A dilute solution of the lactone $C_{10}H_{16}O_3N_2$ in water is kept at room temperature in a vacuum desiccator over phosphorus pentoxide until it is sufficiently concentrated. The acetyl-dipeptide separates in the form of large beautiful crystals. The yield is almost quantitative.

A fairly large amount of this compound was also obtained in this manner from the reaction product of Experiment 2 on α-aminoisobutyric acid. It proved to be as pure as the one prepared from the pure lactone of Experiment 3. The crystals were washed with cold water and dried over phosphorus pentoxide. For analysis they were dried at 62° (chloroform bath) under reduced pressure.

No. 306.
5.245 mg. substance: 10.015 mg. CO_2 and 3.650 mg. H_2O.
6.275 " " : 0.657 cc. N (24°, 776.7 mm.) (Dumas).
$C_{10}H_{18}O_4N_2$ (230.16). Calculated. C 52.14, H 7.88, N 12.17
Found. " 52.07, " 7.78, " 12.32 (Dumas)
" 0 (Van Slyke)
0.2302 gm. substance: 10.00 cc. 0.1 N NaOH (titration, phenolphthalein).
Calculated for 0.001 mol: 10.00 cc. 0.1 N NaOH.

The acetyl-dipeptide melts and decomposes between 223–225° (corrected). Some gas is evolved and a solid new substance is formed. Colorless prismatic needles are deposited in the upper part of the melting point tube.

2. *Preparation from α-Aminoisobutyryl-α-Aminoisobutyric Acid*—A suspension of 3.77 gm. of pure α-aminoisobutyryl-α-aminoisobutyric acid (No. 349; 0.0189 mol, dry) in 20 cc. of glacial acetic acid was gently heated and shaken until all the dipeptide had dissolved. 2.37 gm. of 95 per cent acetic anhydride (0.022 mol, 16 per cent excess) were added; the clear solution was brought to its boiling point and then allowed to cool gradually to room temperature. It was concentrated to dryness under reduced pressure. Crystallization set in at the beginning of this operation. The residue was treated with 1.0 N sodium hydroxide until the mixture was alkaline towards phenolphthalein. The suspension was filtered. There remained a residue of α-aminoisobutyric acid anhydride. It was thoroughly washed with water, then with dilute acid, and again with water. It weighed dry 0.574 gm. (yield, 18 per cent of the theory). The filtrate containing the sodium salt of the acetyl-dipeptide was acidified with 5.0 N HCl and was set aside for slow concentration in a vacuum desiccator over phosphorus pentoxide and soda-lime. After standing for some time, the deposit of crystalline acetyl-dipeptide which had formed was filtered off and well washed with cold water. The filtrate was not further concentrated but was discarded. The crystals weighed dry 1.81 gm. (yield, 42 per cent of the theory). They melted at

224–225° (corrected). An intimate mixture of this substance with that obtained by hydrolysis of the lactone melted at the same temperature.

3. Hydrolysis with Water at 100°—The acetyl-dipeptide is decomposed by water at 100°. A solution of 0.1151 gm. (0.0005 mol) of substance in about 20 cc. of water was boiled for 4 hours under a reflux. Its volume was then brought to 25 cc. The following determinations were made on this solution (No. 320).

4.00 cc. gave 1.83 cc. N at 23° and 763 mm. (Van Slyke, 45 minutes).[12]
10.00 " required 4.13 cc. 0.1 N HCl (Kjeldahl).

$$\frac{\text{NH}_3 \text{ nitrogen}}{\text{Total nitrogen}} = 0.45. \quad \text{Calculated.} \quad 0.50$$

3.00 cc. consumed 0.60 cc. 0.1 N NaOH (titration, phenolphthalein).
Acidity (on 25 cc.): 5.00 cc. 0.1 N NaOH. Calculated. 5.00 cc.[13]

In another experiment a solution of 0.9207 gm. (0.004 mol) of acetyl-dipeptide in 160 cc. of water was boiled for 6 hours under a reflux. It was then evaporated under reduced pressure. The residue was dried and then extracted with hot ethyl acetate. N-Acetyl-α-aminoisobutyric acid was isolated from the filtrate and identified by a mixed melting point determination. The residue of α-aminoisobutyric acid (No. 364) was washed with ethyl acetate and absolute alcohol. It weighed after drying, 0.37 gm. (theory, 0.4124 gm., 0.004 mol).

0.2000 gm. required 19.20 cc. 0.1 N HCl (Kjeldahl).
Found. N 13.44. Calculated. N 13.59

Action of Acetic Anhydride on d,l-Isovaline

The following two experiments were initiated with the purpose of recording the behavior of an amino acid (isovaline), closely related to α-aminoisobutyric acid, under conditions similar to those specified above. As in the case of α-aminoisobutyric acid (Experiments 2 and 3), formation of azlactone undoubtedly takes place to a great extent. This material was similarly very volatile and hence was lost when the excess of acetic anhydride was distilled off. No attempts were made to isolate the remaining solid product of the reaction in a pure state and to establish its nature.

Experiment 1—The procedure was the same as that of Experiment 3 with α-aminoisobutyric acid. A mixture of 11.8 gm. (0.1 mol) of anhydrous *d,l*-isovaline,[16] free of traces of chlorine and of ammonia, and 100 cc. of 95 per cent acetic anhydride (about 0.5 mol) was prepared. The flask was immersed in the oil bath kept at 150°. A short time afterwards, that is before the mixture had started to boil, the amino acid was completely dissolved. The solution was kept boiling for ½ hour, during which time it remained perfectly clear. It was concentrated under reduced pressure. The oily reddish residue was freed in the usual way of acetic anhydride. It solidified only on being kept for a long time in a vaccum desiccator over phosphorus pentoxide, soda-lime, and paraffin chips. It weighed 3.75 gm.

Experiment 2—It was similar in every respect to the foregoing experiment. A small amount of ammonium chloride and a boiling stone, however, were added to the original mixture. These, as far as could be seen, had no effect on the reaction. The amino acid dissolved entirely in the acetic anhydride and no insoluble material was formed on boiling. The solid product of the reaction appeared to be the same as that obtained above.

Azlactone of N-Acetyl-d,l-Phenylmethylaminoacetic Acid

16.5 gm. (0.1 mol) of finely powdered *d,l*-phenylmethylaminoacetic acid dried at 100° (free of ammonium chloride)[17] and 100 cc. of 95 per cent acetic anhydride (about 0.5 mol) were introduced into a 200 cc. round bottom Pyrex flask. The flask was equipped with a reflux condenser and immersed in an oil bath heated to 150°. The mixture began to boil after 10 minutes; the amino acid was completely dissolved after 3 more minutes. The mixture was kept for ½ hour at boiling temperature. It was then concentrated under reduced pressure (water pump). There remained an oily residue. It was taken up in some xylene which was then evaporated off. This operation was repeated twice in order to remove completely any acetic anhydride. The final residue (21.2 gm.) was fractionated through a short Vigreux column sealed to a conical flask. The first fraction contained some xylene. It was in the main composed of pure azlactone. The main fraction, a colorless oil distilling around 119°, under 6 mm. pressure, weighed 13.7 gm. (yield, 72 per cent of the theory). No attempts were made to convert it into a solid material. For identification purposes it was directly transformed into the corresponding acetylamino acid and its amide which are described below.

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[16] Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, 76, 303 (1928).

[17] Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, 76, 308 (1928).

N-Acetyl-d,l-Phenylmethylaminoacetic Acid

A suspension of 13.7 gm. of the above described azlactone in 100 cc. of water was heated to a point where interaction took place rather suddenly. The hydrolysis product separated in the form of a hard beautifully crystalline mass. It was crushed in a mortar. It was thoroughly washed with water and dried. Yield, 13.2 gm. (84 per cent of the theory). Hydration takes place also on prolonged standing of the mixture at room temperature. The acetyl compound was recrystallized twice from hot 20 per cent by weight alcohol. It melted at 202–203° (corrected). The analysis was as follows:

No. 289.
5.825 mg. substance: 13.600 mg. CO_2 and 3.405 mg. H_2O.
6.460 " " : 0.377 cc. N (23°, 767 mm.) (Dumas).
$C_{11}H_{13}O_3N$ (207.12). Calculated. C 63.73, H 6.33, N 6.76
Found. " 63.66, " 6.54, " 6.79

The same compound had been previously obtained,[3] though not in as pure a state, by acetylation of the corresponding amino acid with an excess of acetic anhydride in the presence of pyridine. The product of that operation melted, as reported, at 192–193.5° (uncorrected). It probably contained, at that time, some volatile impurity for after an extensive period of drying, it was now found to melt at a much higher temperature, namely at 198° (uncorrected). A mixture of this material with the purer one now prepared melted at 200° (uncorrected).

N-Acetyl-d,l-α-Aminophenylmethylacetamide

The azlactone of N-acetyl-*d,l*-phenylmethylaminoacetic acid reacts very readily with ammonium hydroxide. It dissolves completely with evolution of heat in an excess of this reagent of sp. gr. 0.90. On cooling, crystallization of the amide takes place. This material was filtered off, washed with some water, dried, and recrystallized from absolute alcohol. The analysis was as follows:

No. 278.

4.540 mg. substance: 10.685 mg. CO_2 and 2.825 mg. H_2O.

6.620 " " : 0.824 cc. N (31°, 754 mm.) (Dumas).

$C_{11}H_{14}O_2N_2$ (206.13).	Calculated.	C 64.04, H 6.85, N 13.59
	Found.	" 64.18, " 6.96, " 13.85

This product was then recrystallized twice from hot 20 per cent by weight alcohol and well dried. It melted at 191–192° (corrected) with slight preliminary sintering.

N-Acetyl-d,l-α-Aminophenylmethylacetyl-α-Aminoisobutyric Acid

1. Preparation—10.0 gm. (0.0525 mol) of azlactone of N-acetyl-*d,l*-phenylmethylaminoacetic acid were added to a hot suspension of 6.20 gm. (0.06 mol, excess 0.0075 mol) of α-aminoisobutyric acid in 60 gm. of glacial acetic acid. The mixture was heated to its boiling point. A clear solution was obtained. It was allowed to cool gradually to room temperature. It was then evaporated to dryness under reduced pressure. The white, crystalline residue was suspended in toluene which was then evaporated off in order to remove all the acetic acid present. The residue was then shaken with water at room temperature. Practically all the acetyl-dipeptide remained undissolved. It was thoroughly washed with cold water which extracted some N-acetyl-*d,l*-phenylmethylaminoacetic acid (1.2 gm. were isolated from the combined filtrate and washings). It was then partly dried in a vacuum desiccator over phosphorus pentoxide, triturated and washed with ether, and then dried. Yield, 12.0 gm. (78 per cent of the theory). This material was dissolved in 17 parts by weight of a mixture of ethyl acetate (35 per cent) and glacial acetic acid (65 per cent by weight). The solution was filtered while hot. 12 parts by weight of ethyl acetate were added to the filtrate and this allowed to cool to room temperature. Crystallization takes place slowly and is complete only after several hours. The crystals were filtered off and washed with an ethyl acetate-acetic acid mixture containing 30 per cent by weight of acid. They retain a rather large amount of solvent which is given off on drying in a vacuum oven at 60°.

No. 359.

4.946 mg. substance: 11.150 mg. CO_2 and 2.960 mg. H_2O.
6.039 " " : 0.509 cc. N (28°, 760.7 mm.).
$C_{15}H_{20}O_4N_2$ (292.18). Calculated. C 61.61, H 6.90, N 9.59
Found. " 61.49, " 6.69, " 9.56

The acetyl-dipeptide melted sharply between 212–213° (uncorrected) and between 214–215° (corrected) with slight evolution of gas.

2. Hydrolysis with Water at 100°—160 cc. of water were added to 1.1685 gm. (0.004 mol) of acetyl-dipeptide and the suspension was boiled under a reflux. After half an hour, all the substance was dissolved. After 4 hours of effective boiling, amino and total nitrogen determinations were made on a portion of this solution (No. 362).

4.00 cc. gave 2.32 cc. N at 29° and 753 mm. (Van Slyke, 45 minutes).[12]
10.00 required 4.73 cc. 0.1 N HCl (Kjeldahl).

Hence, the amino nitrogen:total nitrogen ratio was 0.47 and the substance had suffered hydrolysis to the extent of 94 per cent. The remainder of the solution was kept for 2 more hours at boiling temperature. It was evaporated under reduced pressure. The residue was dried and then extracted with hot ethyl acetate. The filtrate deposited crystals of N-acetyl-phenylmethylaminoacetic acid on cooling. This substance was identified by a mixed melting point determination. The fraction insoluble in ethyl acetate was α-aminoisobutyric acid.

N-Acetyl-Glycyl-α-Aminoisobutyric Acid

1. Preparation—10 cc. of glacial acetic acid were added to 1.60 gm. (0.01 mol) of dry glycyl-α-aminoisobutyric acid.[13] The dipeptide dissolved at room temperature on shaking. 1.19 gm. of 95 per cent acetic anhydride (0.011 mol) were added, the solution was brought to its boiling point, and then allowed to cool gradually to room temperature. It was diluted with toluene and then concentrated to dryness under reduced pressure. Crystallization set in at the very beginning of the distillation. Toluene was again added and then evaporated off in order to remove all the acetic acid. The residue was entirely soluble in hot acetone. The filtrate was concentrated to a small volume, the crystals then deposited were filtered off and washed with acetone. They weighed 0.58 gm. Another fraction was isolated from the mother liquors of crystallization by precipitation with ether. It weighed 0.71 gm. Total, 1.29 gm. (theory, 2.02 gm.). The analysis of the first fraction was as follows:

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[13] Levene, P. A., Steiger, R. E., and Bass, L. W., *J. Biol. Chem.*, **82**, 159 (1929).

No. 355.
4.811 mg. substance: 8.355 mg. CO_2 and 3.115 mg. H_2O.
4.744 " " : 0.584 cc. N (27°, 753.5 mm.) (Dumas).
$C_8H_{14}O_4N_2$ (202.13). Calculated. C 47.50, H 6.98, N 13.86
Found. " 47.35, " 7.24, " 13.88

Each fraction of the acetyl-dipeptide softened at 188° and melted between 189–190° (uncorrected) or between 190–191° (corrected).

2. Hydrolysis with Water at 100°—A solution of 0.1011 gm. (0.0005 mol) of substance in about 20 cc. of water was boiled for 4 hours under a reflux. Its volume was then brought to 25 cc. The following determinations were made on this solution (No. 357).

4.00 cc. gave 0.23 cc. N at 28° and 763 mm. (Van Slyke, 10 minutes)
10.00 " required 4.50 cc. 0.1 N HCl (Kjeldahl).

This gives an amino nitrogen: total nitrogen ratio of 0.05. Hence the substance was hydrolyzed to the extent of 10 per cent.

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STUDIES ON RACEMIZATION

X. ACTION OF ALKALI ON KETOPIPERAZINES AND PEPTIDES

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The various ketopiperazines studied in two previous works[1] on racemization by alkali were unsymmetric. These were composed of an optically active amino acid combined in each case with glycine. It was pointed out that the extent of racemization found, representing the possible limit reached in each case because of hydrolysis, increased with the stability of the ketopiperazine and it was observed that the racemization was more or less complete when the reactions were carried out in alkaline pyridine-water media. In the case of unsymmetric ketopiperazines, undergoing hydrolysis[2] simultaneously with racemization, matters are naturally complicated by the possibility of a ring opening in two different ways, hence by the fact that two secondary reactions of this nature, proceeding at different rates, take place.[3] This point has to be kept in mind in any comparison of the effects produced by the alkali on a series of ketopiperazines of this type. Our observations have now been extended to symmetrically constituted anhydrides composed of two optically active amino acids.

From Table I it is seen that dextro-alanyl-dextro-alanine anhydride is racemized to the extent of 80 per cent under the conditions of the experiment. This limit is reached long before the expiration of the time interval of 48 hours adopted in all our work on ketopiperazines. A corresponding fraction of fully or half active anhydride undergoes hydrolysis to the dipeptides. These, as is shown by the experiment performed on dextro-alanyl-dextro-alanine (see Table I), retain their entire optical activity even on greatly prolonged contact with alkali. Of particular interest is the case of dextro-leucyl-dextro-leucine anhydride. This compound was found to be practically insoluble in water and but sparingly soluble in a pyridine (57 per cent by volume)-water mixture. A solution, 0.04 M in respect to anhydride and 0.08 M in respect to alkali, was prepared and allowed to stand at 25°. Under these conditions no hydrolysis of the anhydride takes place[4] and the progress of racemization can be followed by the decrease in rotation of the solution. From Fig. 1 it is seen that 80 per cent of the anhydride is racemized in the course of about 2 hours and that the racemization is complete after 6 hours (see also Experiment 4, recorded in Table III). A control experiment was made with a similar solution containing no sodium hydroxide. No change in rotation was observed at 25° in the course of several hours. These experiments show conclusively that the racemization values previously observed[1] for other anhydrides in alkaline

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[1] Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **76**, 299 (1928); **86**, 703 (1930).

[2] Levene, P. A., Rothen, A., Steiger, R. E., and Osaki, M., *J. Biol. Chem.*, **86**, 723 (1930).

[3] See Fischer, E., and Schrauth, W., *Ann. Chem.*, **354**, 21 (1907).

TABLE I

Action of 0.2 N Sodium Hydroxide (2 Mols per Mol of Substance) at 25° on Dextro-Alanine Anhydride and on the Corresponding Dipeptide

Compound	Time of action of alkali	$[\alpha]_D^{25}$ of alanine in hydrolysate with 20 per cent HCl (+NaCl)		Racemization
		Treated	Blank	
	days	*degrees*	*degrees*	*per cent*
Dextro-alanyl-dextro-alanine anhydride	2	+2.77	+13.54	80
Dextro-alanyl-dextro-alanine	2 8	$+13.5_0$* $+13.7_6$	$+13.7_9$	0

* Value somewhat low. Hydrolysis not quite complete. The dipeptide is strongly levorotatory in (10 per cent) hydrochloric acid solution; it is only slowly hydrolyzed at 100°. See Fischer, E., *Ber. chem. Ges.*, 39, 466 (1906).

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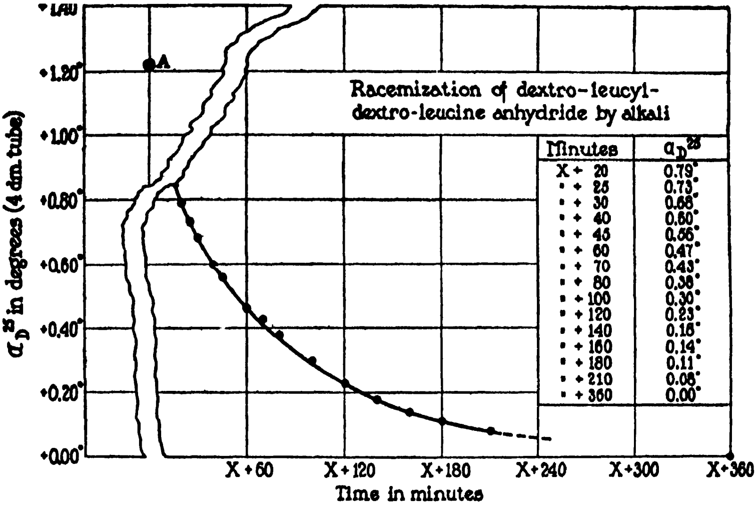
[4] See also Fischer, E., *Ber. chem. Ges.*, 38, 609 (1905) and Fischer, E., and Schrauth, W., *Ann. Chem.*, 354, 27 (1907).

pyridine-water solutions were high due to the fact (established experimentally on glycyl-levo-leucine anhydride[2]) that the rates of hydrolysis of these ketopiperazines were very low in such media. In the case of glycyl-levo-phenylglycine anhydride the rate of racemization was undoubtedly very high. Inasmuch as complete racemization had been observed with this compound it must be inferred that like dextro-leucine anhydride it did not suffer any



Minutes	α_D^{25}
X + 20	0.79°
" + 25	0.73°
" + 30	0.68°
" + 40	0.60°
" + 45	0.56°
" + 60	0.47°
" + 70	0.43°
" + 80	0.38°
" + 100	0.30°
" + 120	0.23°
" + 140	0.16°
" + 160	0.14°
" + 180	0.11°
" + 210	0.08°
" + 360	0.00°

FIG. 1. Showing the decrease in rotation observed with 0.002 mol of dextro-leucyl-dextro-leucine anhydride and 0.004 mol of sodium hydroxide in a pyridine (57 per cent by volume) -water mixture. Volume of the solution, 50.0 cc. at 25°. Concentration of sodium hydroxide, 0.08 N. Concentration anhydride, 0.04 M. *A* is the rotation of the control solution containing the 90 per cent active material used here but no sodium hydroxide (+1.22°). *X* is the very short time interval in which the activity of an optically pure anhydride would drop to that of our starting material.

hydrolysis in the course of the time necessary for the primary reaction to proceed to completion. With these experiments our work on the racemization of ketopiperazines has been brought to the following conclusion. *Ketopiperazines undergo rapid racemization in alkaline solutions. The reaction is complete whenever there is no hydrolysis of the ketopiperazine.*

Polypeptides behave differently. In previous work on the action of alkali on peptides, di-, tri-, and tetrapeptides composed of glycine and levo-alanine were studied.[5] They did not possess the high resistance towards hydrolysis exhibited by the leucine derivatives now examined. The phenomena observed with this latter type of peptides are more readily interpreted.

From Table II it is seen that the dipeptide dextro-leucyl-dextro-leucine is not racemized by 10 equivalents of 1.0 N alkali at 25° and that similarly the two tripeptides listed there retain their full optical activity on prolonged contact with 2 equivalents of 0.2 N sodium hydroxide. In more concentrated alkali (10 equivalents of 1.0 N sodium hydroxide) there is under otherwise identical conditions a slight racemization of these tripeptides. Thus the degree of racemization increases (however slightly) with the concentration of the alkali used and with the length of the peptidic chain.

TABLE II

Racemization of Peptides in Alkaline Solutions at 25°

Compound	Alkali used	$[\alpha]_D^{25}$ of amino acids in hydrolysate with 20 per cent HCl (+NaCl)			Racemization after	
	NaOH	2 days	8 days	Blank	2 days	8 days
	N	*degrees*	*degrees*	*degrees*	*per cent*	*per cent*
Dextro-leucyl-dextro-leucine	1.0*	−15.2	−15.1	−15.2	0	0
Dextro-leucyl-dextro-leucyl-dextro-leucine	0.2†		−15.5	−15.5		0
	1.0*		−14.9			4
Levo-alanyl-dextro-leucyl-dextro-leucine	0.2†		−14.0	−13.9		0
	1.0*		−13.2			5

* 10 mols of NaOH per mol of substance.

† 2 mols of NaOH per mol of substance.

According to Dakin,[6] the racemization of proteins by alkali at low temperatures is due to a keto-enol tautomerism of the

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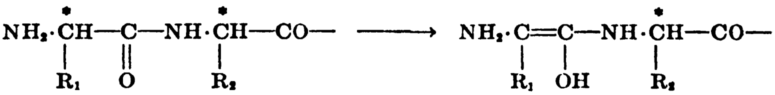
[5] Levene, P. A., and Pfaltz, M. H., *J. Gen. Physiol.*, **8**, 183 (1925); *J. Biol. Chem.*, **68**, 277 (1926); **70**, 219 (1926).

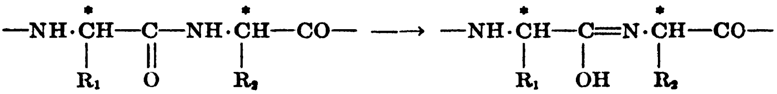
[6] See Dudley, H. W., and Woodman, H. E., *Biochem. J.*, **12**, 341 (1918).

>CH—CO— groups in the protein complex. Wherever this change occurs the asymmetry of that particular grouping will be destroyed. Dakin[7] also expressed the opinion that it appeared likely that the conditions necessary for racemization of an amino acid group required the attachment of other groups to both the amino and the carboxyl radical of this unit. Hence racemization would be observed only with systems composed of at least three amino acids. Our findings substantiate the predictions of Dakin. It may be said, however, that there is no theoretical reason apparent militating against the possibility of a complete racemization in any di- or polypeptide of that terminal amino acid carrying the free amino group.



$$NH_2\cdot\underset{\underset{R_1}{|}}{\overset{*}{C}H}-\underset{\underset{O}{\|}}{C}-NH\cdot\underset{\underset{R_2}{|}}{\overset{*}{C}H}-CO- \longrightarrow NH_2\cdot\underset{\underset{R_1}{|}}{C}=\underset{\underset{OH}{|}}{C}-NH\cdot\underset{\underset{R_2}{|}}{\overset{*}{C}H}-CO-$$

The other type of enolization, not involving the hydrogens attached to the asymmetric carbon atoms, believed to occur in proteins, is the following.



$$-NH\cdot\underset{\underset{R_1}{|}}{\overset{*}{C}H}-\underset{\underset{O}{\|}}{C}-NH\cdot\underset{\underset{R_2}{|}}{\overset{*}{C}H}-CO- \longrightarrow -NH\cdot\underset{\underset{R_1}{|}}{\overset{*}{C}H}-\underset{\underset{OH}{|}}{C}=N\cdot\underset{\underset{R_2}{|}}{\overset{*}{C}H}-CO-$$

The view has been expressed in a previous work[8] that enolization of the second type probably takes place much more readily in polypeptides than in ketopiperazines. An inspection of the schemes above shows that when ready enolization (with formation of more or less stable sodium salts) of the latter type occurs, there is proportionally less racemization to be expected since both these phenomena involve the same central carbonyl groups and hence are antagonistic. A certain minimum concentration of alkali seems to be required to bring about these changes.

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[7] Dakin, H. D., *J. Biol. Chem.*, **13**, 362 (1912–13).

[8] Levene, P. A., Bass, L. W., Steiger, R. E., and Bencowitz, I., *J. Biol. Chem.*, **72**, 815 (1927).

EXPERIMENTAL

1. Preparation of Compounds

Dextro-Alanyl-Dextro-Alanine

This peptide[9] is most conveniently[10] obtained in the following way.[11] Crude *d,l-α*-bromopropionyl-dextro-alanine (from *d,l-α*-bromopropionyl chloride and dextro-alanine) was converted by amination into a mixture of dextro-alanyl-dextro-alanine and levo-alanyl-dextro-alanine. The separation of the two peptides is easily accomplished inasmuch as but the dextro-alanyl-dextro-alanine crystallizes when a large excess of alcohol is added to an aqueous solution of the mixture. The crude peptide was then twice recrystallized in the usual way from water and alcohol.

No. 267.

4.850 mg. substance: 8.030 mg. CO_2 *and 3.325 mg.* H_2O.

4.320 " " : 0.680 cc. (29°, 758.5 mm.).

$C_6H_{12}O_3N_2$ (160.12).	Calculated.	C 44.97, H 7.55, N 17.50
	Found.	" 45.15, " 7.67, " 17.75
	Moisture, 0.39 per cent	

It had the following rotation.

$$[\alpha]_D^{25} = \frac{-5.43° \times 100}{4 \times 6.4047} = -21.2° \text{ (in water)}$$

0.9607 gm., dry, in 15.00 cc. of solution (0.4 M).

Fischer[9] found $[\alpha]_D^{20} = -21.6°$, employing a solution of 0.3523 gm. in water, the weight of the solution being 7.1448 gm. Abderhalden and Delgado y Mier[10] also found $[\alpha]_D^{20} = -21.6°$.

Dextro-Leucyl-Dextro-Leucine

A. Levo-α-Bromoisocapronyl-Dextro-Leucine[12]—A solution of 26.3 gm. (0.2 mol) of dextro-leucine in 100 cc. of 2.0 N sodium hydroxide (0.2 mol) was cooled in an ice-water mixture during treatment with 43 gm. (0.2 mol) of levo-α-bromoisocapronyl chloride (from recrystallized levo-leucine) and 100 cc. of 2.0 N sodium hydroxide (0.2 mol), these reagents being added in equivalent proportions with continuous shaking. 10 cc. of ether and then 52 cc. of 5.0 N hydrochloric acid (0.26 mol) were added to the solution. The bromo compound precipitated as a pasty mass. The mixture was then shaken at room temperature until crystallization took place. The cake was then crushed in a mortar. The crystals were filtered off, washed with ice water, then with petrolic ether (40–60°); they were then washed with water and this was finally displaced by petrolic ether. The bromo compound was dried in a vacuum desiccator. Three such operations were made; the weights of the crude, dried materials were as follows: 55.8 gm. (first operation), 57.2 gm. (second operation), and 55.7 gm. (third operation). The average yield was 91 per cent of the theory. Fischer and Koelker's yield was only 80 per cent.

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[9] Fischer, E., *Ber. chem. Ges.*, **39**, 465 (1906).

[10] Compare Abderhalden, E., and Delgado y Mier, J. J., *Fermentforschung*, **10**, 252 (1928–29).

[11] Fischer, E., and Schulze, A., *Ber. chem. Ges.*, **40**, 945, 952, 954 (1907).

[12] Compare with the indications of Fischer, E., and Koelker, A. H., *Ann. Chem.*, **354**, 39 (1907). See also Fischer, E., *Ber. chem. Ges.*, **39**, 2918 (1906).

B. Dextro-Leucyl-Dextro-Leucine[12]—A mixture of 168.5 gm. of crude levo-α-bromoisocapronyl-dextro-leucine with 8 times this weight of ammonium hydroxide of sp. gr. of 0.90 was kept at room temperature for 6 days. The solution was diluted with water, clarified with norit, and concentrated under reduced pressure. It yielded thus several fractions of dipeptide. A large volume of alcohol was added to the filtrate of the last fraction and the mixture was evaporated to dryness. The residue was extracted with cold absolute alcohol. The dipeptide which had remained insoluble was filtered off, washed with absolute alcohol and with benzene. The various fractions of dipeptide were combined. Yield of air-dried material containing water of crystallization, 100 gm. This crude material was dissolved in 18.5 parts (by weight) of 85 per cent by weight alcohol at boiling temperature. Norit was added and the solution filtered while hot. The filtrate was cooled for 60 hours in ice. The crystals were filtered off, washed with absolute alcohol, and dried in the air. Yield, 51 gm. They were again recrystallized from boiling 85 per cent by weight alcohol in which, curiously enough, they were much less soluble than the original material. The solution saturated at boiling temperature was rapidly filtered with suction. Immediately crystallization took place; the mixture was then cooled in ice. The crystals were washed with absolute alcohol, dried in the air, and then kept for several months in a vacuum desiccator over phosphorus pentoxide. They weighed 25.3 gm. (No. 315). This substance contained 7.74 per cent of moisture. This was established in the following way. When heated for 2½ hours to 100° under diminished pressure, the moist dipeptide lost 7.19 per cent of water. The drying was then interrupted inasmuch as according to Fischer the peptide is after this time partly converted into the corresponding anhydride. Two analyses of the material thus obtained were performed.

3.100 mg. substance: 6.655 mg. CO_2 and 2.745 mg. H_2O.
3.558 " " : 7.660 " " " 3.190 " "
$C_{12}H_{24}O_4N_2$ (244.21). Calculated. C 58.97, H 9.91
Found. " 58.54, " 9.90
" 58.70, " 10.00

It contained, therefore (C 58.62 per cent, mean), 99.41 per cent of dry dipeptide. Hence, the original substance (No. 315), contained 92.26 per cent of dry dipeptide or 7.74 per cent of water. The rotation was as follows:

$$[\alpha]_D^{25} = \frac{+2.51^\circ \times 100}{4 \times 4.5273} = +13.86^\circ \text{ (in 1.0 N NaOH)}$$

0.7371 gm. of moist dipeptide (0.6800 gm., dry) in 15.02 cc. of solution.

This material (dry) contained 98 per cent of dextro-leucine in combined form (see hydrolysis Experiment 15, recorded in Table IV). Fischer and Koelker[13] found $[\alpha]_D^{20} = +13.16^\circ$ (±0.2°), employing a solution of 0.3810 gm. of moist dipeptide (0.3193 gm., dry) in 1.0 N sodium hydroxide, the weight of the solution being 3.5868 gm. Fischer[14] found for levo-leucyl-levo-leucine $[\alpha]_D^{20} = -13.43^\circ$ (±0.4°), employing a solution of 0.1628 gm. of moist dipeptide (0.1512 gm., dry) in 1.0 N sodium hydroxide, the weight of the solution being 3.4877 gm. Abderhalden and Fleischmann[15] found for levo-leucyl-levo-leucine $[\alpha]_D^{20} = -13.95^\circ$ in 1.0 N sodium hydroxide.

Another sample of dipeptide (No. 263) of another preparation, after having been kept for a long period in a vacuum desiccator over phosphorus pentoxide, was then found to contain exactly 1 mol of water of crystallization. It lost 5.72 per cent of water on heating for 2½ hours to 100°.

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[13] Fischer, E., and Koelker, A. H., *Ann. Chem.*, **354**, 48 (1907).

[14] Fischer, E., *Ber. chem. Ges.*, **39**, 2919 (1906).

[15] Abderhalden, E., and Fleischmann, R., *Fermentforschung*, **9**, 526 (1926–28).

4.445 mg. substance: 9.495 mg. CO_2 and 3.850 mg. H_2O.
$C_{12}H_{24}O_3N_2$ (244.21). Calculated. C 58.97, H 9.91
Found. " 58.25, " 9.69

The dried material thus contained 98.78 per cent of dipeptide and hence the original substance (No. 263) 93.13 per cent of dry dipeptide or 6.87 per cent of water.

$C_{12}H_{24}O_3N_2 + 1\ H_2O$ (262.23). Calculated. H_2O 6.87 per cent
Found. " 6.87 " "

Dextro-Alanyl-Dextro-Alanine Anhydride

It was prepared by heating the methyl ester of dextro-alanine for 12 hours at 100°.[16] The crude product was thoroughly washed with ether. It was then purified by recrystallization from 10 times its weight of boiling water.

No. 313.
4.380 mg. substance: 8.135 mg. CO_2 and 2.795 mg. H_2O.
3.060 " " : 0.537 cc. N (25°, 756.2 mm.).
$C_6H_{10}O_2N_2$ (142.10). Calculated. C 50.67, H 7.09, N 19.72
Found. " 50.56, " 7.14, " 19.98
Moisture, 0.45 per cent

It had the following rotation.

$$[\alpha]_D^{25} = \frac{-1.57° \times 100}{4 \times 1.4214} = -27.6° \text{ (in water)}$$

0.2132 gm., dry, in 15.00 cc. of solution (0.1 M).

Fischer[16] found for dextro-alanyl-dextro-alanine anhydride prepared from the ester of dextro-alanyl-dextro-alanine, $[\alpha]_D^{20} = -28.8° (\pm 0.5°)$, employing a solution of 0.2741 gm. in water, the weight of the solution being 12.8787 gm., and the constant value of $[\alpha]_D^{20} = -27°$ for the anhydride obtained by heating the methyl ester of dextro-alanine for 12 hours at 100°.

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[16] Fischer, E., *Ber. chem. Ges.*, **39**, 468 (1906).

Dextro-Leucyl-Dextro-Leucine Anhydride

It was prepared from dextro-leucyl-dextro-leucine (an optically impure material was used) by the procedure established by Fischer[17] for levo-leucyl-levo-leucine anhydride. It was purified by recrystallization from boiling alcohol. Two large fractions were obtained.[18] The analysis of the first fraction was as follows:

No. 316.
4.860 mg. substance: 11.372 mg. CO_2 and 4.125 mg. H_2O.
4.960 " " : 0.546 cc. N (28°, 757.3 mm.).
$C_{12}H_{22}O_2N_2$ (226.20). Calculated. C 63.66, H 9.80, N *12.39*
Found. " *63.81*, " *9.90*, " *12.43*
Moisture, 0.37 per cent

This material had the following rotation.

$$[\alpha]_D^{25} = \frac{+4.02^\circ \times 100}{4 \times 2.2660} = +44.3_5^\circ \text{ (in glacial acetic acid)}$$

0.3399 gm., dry, in 15.00 cc. of solution.

It contained from 95 to 96 per cent of pure dextro-leucine anhydride, inasmuch as (see for a check hydrolysis Experiment 14, recorded in Table IV) Fischer and Koelker[18] $[\alpha]_D^{20} = +48.67^\circ$ ($\pm 0.3^\circ$), employing a solution of 0.2476 gm. in glacial acetic acid, the weight of the solution being 2.8424 gm. Other preparations by these authors had lower rotations.

Dextro-Leucyl-Dextro-Leucyl-Dextro-Leucine

A. Levo-α-Bromoisocapronyl-Dextro-Leucyl-Dextro-Leucine—A solution of 29.4 gm. (dry, 0.12 mol) of dextro-leucyl-dextro-leucine (34.8 gm. of dipeptide containing 15 per cent of moisture) in 120 cc. of 1.0 N sodium hydroxide (0.12 mol) was cooled in an ice-water mixture during treatment with 33.4 gm. (0.156 mol) of levo-α-bromoisocapronyl chloride (from recrystallized levo-leucine) and 180 cc. of 1.0 N sodium hydroxide (0.18 mol), these reagents being added in equivalent proportions with continuous shaking. A crystalline precipitate of the bromo compound was obtained at once on addition of 46 cc. of 5.0 N hydrochloric acid (0.23 mol). It was washed with cold water and then with petrolic ether. It weighed 46 gm. (yield, 91 per cent of the theory). This crude material was recrystallized three times in the following way. Water was added to the hot alcoholic solution to incipient turbidity. The bromo compound crystallized on cooling. It was converted as described below into the tripeptide fraction No. 268. The bromo compound contained in the mother liquors of crystallization of the preceding was recovered; it was also aminated, but separately. It yielded the tripeptide fraction No. 269.

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[17] Fischer, E., *Ber. chem. Ges.*, **39**, 2920 (1906).

[18] Fischer, E., and Koelker, A. H., *Ann. Chem.*, **354**, 49 (1907).

B. Dextro-Leucyl-Dextro-Leucyl-Dextro-Leucine. Main Fraction—A mixture of 21.5 gm. of the recrystallized bromo compound with 10 times this weight of ammonium hydroxide of sp. gr. 0.90 was kept at 37° for 3 days, during which time it was occasionally shaken. The solution was diluted with an equal volume of water; it was clarified with norit, and evaporated to dryness under reduced pressure, octyl alcohol being added from time to time to prevent frothing. The residue was suspended in lukewarm absolute alcohol; it was filtered off after cooling, and thoroughly washed with absolute alcohol; it was then washed successively with ether, water, alcohol, and finally with ether. This crude tripeptide weighed 15 gm. It was suspended in 450 gm. of absolute alcohol; 15 gm. of ammonium hydroxide of sp. gr. 0.90 and then norit were added. The filtered solution was concentrated under reduced pressure. As the ammonia was removed, the tripeptide precipitated. The suspension was left to cool in the ice box. The crystals were washed with alcohol, then with ether and air-dried; they weighed 15 gm. This material was once more purified in the same way. A sample of the substance thus obtained (No. 268) was dried for exactly 2½ hours at 100° under diminished pressure (loss of weight, 4.10 per cent) and two analyses of this partly dried material (*cf.* case of dextro-leucyl-dextro-leucine) were performed.

3.577 mg. substance: 7.615 mg. CO_2 and 3.220 mg. H_2O.
4.232 " " : 9.037 " " " 3.845 " "
$C_{18}H_{35}O_4N_3$ (357.31). Calculated. C 60.45, H 9.87
Found. " 58.06, " 10.07
" 58.23, " 10.16

The dried material contained, therefore (C 58.15 per cent, mean), 96.20 per cent of dry tripeptide. Hence the original substance

(No. 268) contained 92.26 per cent of dry tripeptide or 7.74 per cent of moisture. The rotation of the tripeptide was as follows:

$$[\alpha]_D^{25} = \frac{+11.87^\circ \times 100}{4 + 6.5440} = +45.4^\circ \text{ (in 1.010 N NaOH)}$$

0.9816 gm., dry, in 15.00 cc. of solution.

Abderhalden and Fleischmann[19] found for levo-leucyl-levo-leucyl-levo-leucine $[\alpha]_D^{20} = -51.36^\circ$, employing a solution of 0.3115 gm. in 1.0 N sodium hydroxide, the volume of the solution being 10 cc. *Thus at first sight, if it is assumed that the rotation of the optically pure tripeptide* (composed of dextro acids) is $[\alpha]_D^{20} = +51.36^\circ$, it would seem that our material was only 88 per cent active. That such a comparison is not permissible is clearly shown by the result of the hydrolysis experiment (Experiment 16, recorded in Table IV). The rotation of the leucine contained in the acid hydrolysate of this tripeptide was $[\alpha]_D^{25} = -15.5^\circ$. Since Fischer and Warburg[20] found for pure levo-leucine $[\alpha]_D^{20} = +15.8^\circ$, in 20 per cent hydrochloric acid, the tripeptide contained 99 per cent of dextro-leucine.

Second Fraction (*No. 269*)—It was prepared from a seemingly less pure levo-α-bromoisocapronyl-dextro-leucyl-dextro-leucine (see that paragraph). It was purified in the same way as the main fraction above. It had the following rotation.

$$[\alpha]_D^{25} = \frac{+11.97^\circ \times 100}{4 \times 6.5087} = +46.0^\circ \text{ (in 1.010 N NaOH)}$$

0.9763 gm., dry, in 15.00 cc. of solution (concentration determined by Kjeldahl. 5.00 cc. of this solution contained 0.7655 gm. of nitrogen).

Levo-α-Bromopropionyl-Dextro-Leucyl-Dextro-Leucine

A solution of 29.4 gm. (dry, 0.12 mol) of dextro-leucyl-dextro-leucine (34.8 gm. of dipeptide containing 15 per cent of moisture) in 120 cc. of 1.0 N sodium hydroxide (0.12 mol) was cooled in an ice-water mixture during treatment with 27 gm. (0.156 mol) of levo-α-bromopropionyl chloride (from recrystallized dextro-alanine) and

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[19] Abderhalden, E., and Fleischmann, R., *Fermentforschung*, 9, 528 (1926–28).

[20] Fischer, E., and Warburg, O., *Ber. chem. Ges.*, 38, 4004 (1905).

180 cc. of 1.0 N sodium hydroxide (0.18 mol), these reagents being added in equivalent proportions with continuous shaking. 46 cc. of 5.0 N hydrochloric acid were then added. The precipitate of crude bromo compound weighed after drying 40 gm. (yield, 88 per cent of the theory). It was purified in the following way. 40 gm. were dissolved in 210 gm. of hot 35 per cent by weight alcohol. The crystals obtained on cooling in ice water were washed with 35 per cent alcohol. They weighed 38.5 gm. These were dissolved in 158 gm. of 45 per cent by weight alcohol and gave 36 gm. of a purer material. This was dissolved in 216 gm. (6 parts) of 55 per cent by weight alcohol. It gave 28 gm. which finally were dissolved in 168 gm. (6 parts) of 50 per cent by weight alcohol. 25 gm. of bromo compound were obtained. It was dried in a vacuum desiccator over phosphorus pentoxide and soda-lime. It melted and decomposed at 180° (uncorrected).

No. 270.
4.065 mg. substance: 7.135 mg. CO_2 and 2.690 mg. H_2O.
0.1000 gm. " : 5.05 cc. 0.1 N HCl (Kjeldahl).
0.1120 " " : 0.0552 gm. AgBr (Carius).
$C_{15}H_{27}O_4N_2Br$ (379.16). Calculated. C 47.47, H 7.18, N 7.39, Br 21.08
Found. " 47.86, " 7.40, " 7.07, " 20.97
Moisture, none

It had the following rotation.

$$[\alpha]_D^{25} = \frac{+14.06° \times 100}{4 \times 7.5833} = +46.35° \text{ (in absolute alcohol)}$$

1.1375 gm. in 15.00 cc. of solution (0.2 M).

Levo-Alanyl-Dextro-Leucyl-Dextro-Leucine

A mixture of 21.5 gm. of purified bromo compound, $[\alpha]_D^{25} = +46.35°$, with 10 times this weight of ammonium hydroxide of sp. gr. 0.90 was kept at 37° for 3 days, during which time it was occasionally shaken. The solution was diluted with an equal volume of water. It was clarified with norit and evaporated to dryness under reduced pressure, octyl alcohol being added from time to time to prevent frothing. The residue was dried by reevaporation with absolute alcohol; the suspended material was filtered off and was washed with absolute alcohol; it was then washed successively with water, alcohol, and finally with ether. This crude tripeptide weighed 14 gm. It was suspended in 420 gm. of absolute alcohol, 14 gm. of ammonium hydroxide of sp. gr. 0.90 were added, and the mixture was shaken at room temperature until practically all had dissolved. Norit was then added and the filtered solution was concentrated under reduced pressure. As the ammonia was removed, the tripeptide precipitated as a gel. It became crystalline when left to stand overnight in the ice box. The crystals were filtered off, washed with absolute alcohol and with anhydrous ether, and dried in the air; yield, 11 gm. This material was once more purified in the same way; yield, 10 gm.

No. 271.

3.540 mg. substance: 7.435 mg. CO_2 and 2.895 mg. H_2O.
6.275 " " : 0.742 cc. N (23°, 752 mm.).
$C_{15}H_{29}O_4N_3$ (315.26). Calculated. C 57.10, H 9.27, N 13.33
Found. " 57.27, " 9.15, " 13.49
Moisture, 1.32 per cent (100°)

It had the following rotation.

$$[\alpha]_D^{25} = \frac{+7.82° \times 100}{4 \times 3.1527} = +62.0° \text{ (in 1.010 N NaOH)}$$

0.4729 gm., dry, in 15.00 cc. of solution (0.1 M).

Another fraction of tripeptide was prepared by amination of the bromo compound contained in the combined mother liquors of crystallization of the purified product. It was purified in the same way as the main fraction. The analysis was as follows:

No. 272.

4.760 mg. substance: 9.980 mg. CO_2 and 3.860 mg. H_2O.
4.425 " " : 0.520 cc. N (24° 754.4 mm.).
$C_{15}H_{29}O_4N_3$ (315.26). Calculated. C 57.10, H 9.27, N 13.33
Found. " 57.17, " 9.07, " 13.40
Moisture, 0.30 per cent

It had the following rotation.

$$[\alpha]_D^{25} = \frac{+7.84° \times 100}{4 \times 3.1527} = +62.2° \text{ (in 1.010 N NaOH)}$$

0.4729 gm., dry, in 15.00 cc. of solution (0.1 M).

TABLE III

Action of Sodium Hydroxide at 25° on Ketopiperazines, Dipeptides, and Tripeptides

Compound	Dextro-alanyl-dextro-alanine anhydride	Dextro-alanyl-dextro-alanine	Dextro-leucyl-dextro-leucine anhydride	Dextro-leucyl-dextro-leucine	Dextro-leucyl-dextro-leucyl-dextro-leucine	Levo-alanyl-dextro-leucyl-dextro-leucine
Experiment No..................	1	2 and 3	4	5 and 6	7 and 8	9 and 10
Amount of substance (0.0025 mol, dry), *gm.*	0.3553	0.4003 (2) 0.4003 (3)	0.4524 (0.002 mol)	0.6106 (5) 0.6106 (6)	0.8933 (7) 0.8933 (8)	0.7882 (9) 0.7882 (10)
Reagent used	∽ 19 cc. water + 5.00 cc. 1.0 N NaOH (0.005 mol) + water. Volume, 25.0 cc. (25°)		28.50 cc. pyridine +16 cc. water + 4.00 cc. 1.0 N NaOH (0.004 mol) + water. Volume, 50.0 cc.	25.0 cc. 1.0 N NaOH (0.025 mo	∽ 19 cc. water + 5.00 cc. 1.0 N NaOH (0.005 mol) + water. Volume, 25.0 cc. (25°) 25.0 cc. 1.0 N NaOH (0.025 mol) for Experiments 8 and 10	
Action of alkali at 25°, *days*	2	2 (2) 8 (3)	2	2 (5) 8 (6)	8 (7) 8* (8)	8 (9) 8 (10)
Total N in hydrolysate with 20 cc. of 20 per cent HCl, *gm. per 100 cc.*	0.3407_6	0.3407_6 (2) 0.3371 (3)	0.2744	0.3374 (5) 0.3318 (6)	0.5191_2 (7) 0.4916_8 (8)	0.5076_4 (9) 0.4916_8 (10)
$\alpha_1{}^{25}_{D}$ (4 dm. tube), *degrees*	+0.24	+1.17 (2) +1.18 (3)	0.00	−1.92 (5) -1.87_5 (6)	−3.01 (7) −2.75 (8)	−2.38 (9) −2.17 (10)
$[\alpha_1]^{25}_{D}$ of amino acids in solution, *degrees*	+2.77	$+13.5_0$ (2) $+13.7_6$ (3)	0.0_6	-15.2_0 (5) -15.1_9 (6)	-15.4_9 (7) -14.9_4 (8)	-14.0_2 (9) -13.2_9 (10)

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* The solution deposited long needles of an unidentified substance. Compare Abderhalden, E., and Fleischmann, R., *Fermentforschung*, 9, 532 (1926–28).

TABLE IV

Hydrolysis of Ketopiperazines, Dipeptides, and Tripeptides by Hydrochloric Acid at 100°

Compound	Dextro-alanyl-dextro-alanine anhydride	Dextro-alanyl-dextro-alanine	Dextro-leucyl-dextro-leucine anhydride	Dextro-leucyl-dextro-leucine	Dextro-leucyl-dextro-leucyl-dextro-leucine	Levo-alanyl-dextro-leucyl-dextro-leucine
Experiment No.	11 and 12	13	14	15	16	17 and 18
Amount of substance (0.0025 mol, dry), *gm.*	0.3553 (11) 0.3553 (12)	0.4003	0.4524 (0.002 mol)	0.6106	0.8933	0.7882 (17) 0.7882 (18)
Amount of NaCl added, *gm.* " " " " *mol*	0.2923 0.005	0.2923 0.005	0.2339 0.004	0.2923 0.005	0.2923 0.005	0.2923 0.005
Total N in hydrolysate with 20 cc. 20 per cent HCl, *gm. per 100 cc.*	0.3455_2 (11) 0.3449_6 (12)	0.3435_6	0.2758	0.3435_6	0.5283_6	0.5073_6 (17) 0.5157_6 (18)
$\alpha_0{}^{25}_{D}$ (4 dm. tube), *degrees*	+1.19 (11) +1.19 (12)	$+1.20_5$	−1.48	-1.95_5	−3.07	−2 36 (17) −2.38 (18)
$[\alpha_0]^{25}_{D}$ of amino acids in solution, *degrees*	$+13.5_4$ (11) $+13.5_6$ (12)	$+13.7_9$	-14.3_3	-15.2_0	-15.5_2	-13.9_1 (17) -13.8_0 (18)

2. Action of Sodium Hydroxide on Ketopiperazines, Dipeptides, and Tripeptides (Racemization)

Alkaline solutions of the compounds prepared were allowed to stand for certain lengths of time in a thermostat kept at 25°. All the specific data are recorded in Table III. The solutions were then acidified and concentrated to dryness under reduced pressure in the way indicated in a previous work.[1] The dry residues were taken up in 20 cc. of 20 per cent hydrochloric acid. The test-tubes were sealed and immersed for 24 hours in a steam-heated water bath at 100°. The solutions or suspensions obtained were filtered in order to remove insoluble particles or undissolved sodium chloride. The total nitrogen was determined on 5 cc. and the amino nitrogen on 1 cc. of solution. For all other details of technique, see the preceding paper[1] of this series.

The extent of racemization by alkali is given in per cent by the ratio

$$\frac{[\alpha_0]_D^{25} - [\alpha_1]_D^{25}}{[\alpha_0]_D^{25}} \times 100$$

The values $[\alpha_0]_D^{25}$ of the blanks are given in Table IV.

3. Action of Hydrochloric Acid on Ketopiperazines, Dipeptides, and Tripeptides (Hydrolysis)

The various compounds prepared were hydrolyzed with 20 per cent hydrochloric acid in the presence of sodium chloride. The sealed tubes were immersed for 24 hours in a steam-heated water bath at 100°. All the experimental data are recorded in Table IV. The values $[\alpha_0]_D^{25}$ were thus obtained.

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THE RING STRUCTURE OF NORMAL METHYLRIBOSIDE

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(Received for publication, June 29, 1931)

It has been shown that oxidation of the normal trimethyl derivatives of xylose,[1] arabinose,[2] and lyxose[3] (or simultaneous hydrolysis and oxidation of their methyl glycosides), by concentrated nitric acid, leads in each case to the formation of a trimethoxyglutaric acid. This implies that in each of these three trimethyl pentoses the methoxyl groups are attached to the second, third, and fourth carbon atoms and that the oxygen of the ring must be attached to the fifth carbon atom; *i.e.*, they are all pyranose derivatives.

Similar experimental methods have now been applied to determine the structure of normal methylriboside and definite proof has been obtained to the effect that this substance and its derivatives are of the pyranose type and thus conform to the general rule followed by normal sugar derivatives.

The normal trimethyl ribose required for the oxidation experiments was prepared by methylating a syrupy methylriboside which had been subjected to prolonged treatment with 1.5 per cent methyl alcoholic hydrogen chloride to insure complete transformation of any methylribofuranoside.

Hydrolysis of this trimethyl methylriboside gave crystalline trimethyl ribose; m.p., 85°. The constitution of this substance as 2, 3, 4-trimethyl ribose followed at once from a study of its oxidation by nitric acid; and also from the nature of the trimethyl ribonolactone obtained from it by the action of bromine water.

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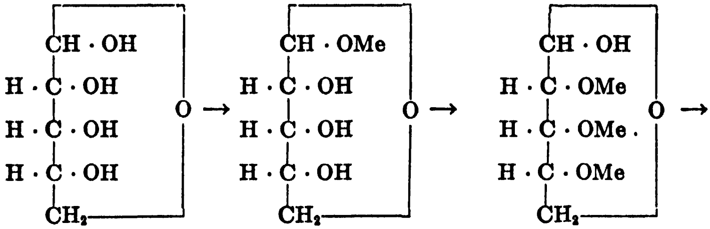
[1] Hirst, E. L., and Purves, C. B., *J. Chem. Soc.*, **123**, 1352 (1923).

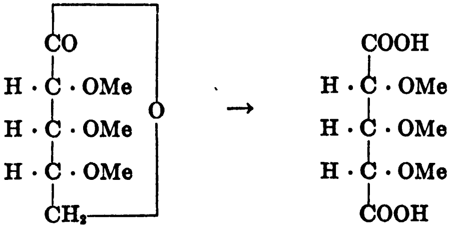
[2] Hirst, E. L., and Robertson, G. J., *J. Chem. Soc.*, **127**, 358 (1925). McOwan, G., *J. Chem. Soc.*, 1747 (1926).

[3] Hirst, E. L., and Smith, J. A. B., *J. Chem. Soc.*, 3147 (1928).

The lactone displayed the behavior typical of a δ-lactone, since polarimetric observations during its hydrolysis in aqueous solution showed that equilibrium was attained in about 191 hours and that some 44 per cent of the lactone then remained unchanged. It is similar in its rate of hydrolysis to tetramethyl δ-mannonolactone and trimethyl δ-rhamnonolactone to which it is related in the configuration of carbon atoms (2) and (3). In addition, this lactone affords a further striking example of the profound influence[4] exerted by the solvent upon the specific rotation of methylated sugar lactones having *cis* methoxyl groups attached to carbon atoms (2) and (3). When oxidized with concentrated nitric acid the sugar gave exclusively *i*-ribotrimethoxyglutaric acid, which was isolated in the form of its dimethyl ester.



d-Ribose Methylriboside 2,3,4-Trimethylribose



Trimethyl δ-ribonolactone *i*-Ribotrimethoxyglutaric acid

EXPERIMENTAL

Preparation of Trimethyl Methylriboside—Methylriboside was prepared as previously described.[5] 10.9 gm. of syrupy methylriboside were dissolved in 20 cc. of acetone and treated in the usual manner with 44 cc. of dimethyl sulfate and 86 cc. of 30 per cent aqueous sodium hydroxide at 55–70°, the temperature finally being raised to 100° for 30 minutes. Methylation of the product was completed by the use of Purdie's reagents and the resulting non-reducing syrup (13.1 gm.) was distilled giving two fractions.

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[4] Haworth, W. N., Hirst, E. L., and Smith, J. A. B., *J. Chem. Soc.*, 2659 (1930).

[5] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **92**, 109 (1931).

(*a*) 9 gm. of a colorless, very mobile liquid boiling at 54° at 0.05 mm. (bath temperature, 73–77°), N_D^{21}, 1.4473.

It had the following composition.

4.810 mg. substance: 9.220 mg. CO_2 and 3.840 mg. H_2O.
6.896 " " : 30.900 " AgI.
$C_9H_{18}O_5$. Calculated. C 52.4, H 8.8, OMe 60.2
Found. " 52.3, " 8.9, " 59.2

Its rotation was as follows:

$$[\alpha]_D^{24} = \frac{-1.75° \times 100}{2 \times 2.501} = -35.0° \text{ (in water)}$$

$$[\alpha]_D^{24} = \frac{-1.12° \times 100}{2 \times 2.316} = -24.2° \text{ (in methyl alcohol)}$$

The substance, which possessed typical glucosidic properties, resisted all attempts to crystallize it.

(*b*) 3.5 gm. of a colorless mobile liquid boiling at 55–60° at 0.06 mm. (bath temperature, 76–98°), N_D^{23}, 1.4470.

4.220 mg. substance: 18.397 mg. AgI.
$C_9H_{18}O_5$. Calculated. OMe 60.2. Found. OMe 57.6

The second fraction was evidently not completely methylated, so was not used in the following work.

Preparation of 2, 3, 4-Trimethyl Ribose—4.0 gm. of trimethyl methylriboside were dissolved in 100 cc. of N/15 hydrochloric acid. The absence of furanose derivatives was shown by the negligible rate of hydrolysis at 90°. Sufficient concentrated hydrochloric acid was now cautiously added, with vigorous stirring, to raise the concentration to approximately 6 per cent hydrochloric acid and the resulting solution was heated at 98°. In the course of 1 hour the specific rotation altered from $[\alpha]_D^{24} = -35.7°$ to a constant value $[\alpha]_D^{24} = -10.0°$. The hydrochloric acid was then partly neutralized with lead carbonate and the neutralization completed with barium carbonate. The mixture was filtered, the filtrate evaporated to dryness, and the *trimethyl ribose* was extracted by means of chloroform. Removal of solvent gave 3.5 gm. of syrupy product. This was dissolved in dry ether and on cautious addition of petroleum ether (30–40°) trimethyl ribose commenced crystallizing immediately in clusters of long fine needles, crystallization being complete in a few minutes; m.p., 80°. It was recrystallized from a mixture of ether and petroleum ether at 0°; m.p., 85–86°. Weight, 2.82 gm. It was strongly reducing towards boiling Fehling's solution and had a bitter taste. It had the following composition.

4.609 mg. substance: 8.440 mg. CO_2 and 3.457 mg. H_2O.
4.155 " " : 15.450 " AgI.

$C_8H_{16}O_5$.	Calculated.	C 49.97,	H 8.4,	OMe 48.45
	Found.	" 49.94,	" 8.4,	" 49.08

Its rotation in water was

$$[\alpha]_D^{27} = \frac{-1.11^\circ \times 100}{2 \times 1.074} = -51.7^\circ \text{ (initial)}$$

$$[\alpha]_D^{27} = \frac{-0.86^\circ \times 100}{2 \times 1.074} = -40.0^\circ \text{ (final)}$$

In another hydrolysis experiment under the same conditions 3.82 gm. of trimethyl methylriboside gave 2.97 gm. of crystalline trimethyl ribose (83.4 per cent yield). During the hydrolysis the specific rotation changed from −34.4° to −9.7°.

Since the yield was not as high as might be expected and since the final rotation of the hydrolysis mixture did not correspond to the equilibrium rotation of pure trimethyl ribose, it seemed possible that decomposition, similar to that encountered by Carruthers and Hirst[6] during the hydrolysis of trimethyl methylxyloside, might have occurred owing to the use of too concentrated an acid solution and too elevated a temperature. It was therefore decided to use 4 per cent hydrochloric acid as hydrolyst,[7] and to follow the course of the reaction at 85° both by observation of the specific rotation and by quantitative estimation of the free sugar present after various time intervals.

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[6] Carruthers, A., and Hirst, E. L., *J. Chem. Soc.*, **121**, 2299 (1922).

[7] Phelps, F. P., and Purves, C. B., *J. Am. Chem. Soc.*, **51**, 2443 (1929).

Approximately 5 gm. of trimethyl methylriboside were weighed accurately into a volumetric flask and made to 100 cc. with 4 per cent hydrochloric acid. The specific rotation of the solution was then observed after which it was heated under a reflux condenser in a bath at 85°. At appropriate times the solution was rapidly cooled in ice to room temperature and its specific rotation again observed. The amount of free sugar present in an aliquot part was then determined by the method of Willstätter and Schudel[8] as modified by Goebel,[9] the sample first being rendered neutral by titration with 0.1 N sodium hydroxide. That no appreciable evaporation occurred, during the period of heating under the reflux, was indicated by the constancy of this initial titer. The results are given in Table I.

TABLE I

Course of the Hydrolysis of Trimethyl Methylriboside

Time	$[\alpha]_D^{27}$	Trimethyl pentose formed
hrs.	*degrees*	*per cent*
0.0	−43.6	0.0
0.5	−41.1	
1.5	−33.6	
2.0	−31.0	86.5
2.5	−28.5	90.7
3.0	−26.6	93.3
3.5	−24.7	98.1

From these results it seems probable that the presence of hydrochloric acid considerably modifies the specific rotation of trimethyl ribose in water.

Oxidation of Crystalline 2, 3, 4-Trimethyl Ribose—2.82 gm. of crystalline trimethyl ribose were dissolved in 20 cc. of water and 3 cc. of bromine were added, in portions of 0.5 cc., during 4 days at 38°. The excess bromine was then removed by aeration, silver oxide was added until all the mineral acid had been neutralized, the mixture was filtered, and the silver salts well washed with hot water. To the combined aqueous solution was added dilute hydrochloric acid from a burette until all the silver in solution had been precipitated. The mixture was filtered and the filtrate evaporated to a thick syrup under diminished pressure at 30°. The product was heated at 100° at 15 mm. for 5 hours in order to complete lactonization and then distilled at high vacuum. The main fraction (weight, 1.5 gm.) boiled at 93–95° at 0.05 mm. (bath temperature, 116–118°). It was quite mobile and had N_D^{25}, 1.4572.

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[8] Willstätter, R., and Schudel, G., *Ber. chem. Ges.*, **51**, 780 (1918).

[9] Goebel, W. F., *J. Biol. Chem.*, **72**, 812 (1927).

Its analysis was as follows:

3.366 mg. substance: 6.284 mg. CO_2 and 2.201 mg. H_2O.
4.564 " " : 16.800 " AgI.

$C_8H_{14}O_5$.	Calculated.	C 50.50, H 7.4, OMe 48.96
	Found.	" 50.91, " 7.3, " 48.63

100 mg. substance required 5.36 cc. 0.1 N NaOH.
$C_8H_{14}O_5$. Calculated. 5.26 cc.

The substance displayed the following specific rotations.

$$[\alpha]_D^{27} = \frac{+1.28° \times 100}{2 \times 0.924} = +69.3° \text{ (in chloroform)}$$

$$[\alpha]_D^{27} = \frac{+2.02° \times 100}{2 \times 0.885} = +114.1° \text{ (in dry ether)}$$

$$[\alpha]_D^{27} = \frac{+1.77° \times 100}{2 \times 1.036} = +85.4° \text{ (in benzene)}$$

The rotation of the free acid was determined in the usual manner by forming the sodium salt, adding the equivalent amount of hydrochloric acid, and observing the rotation immediately. Calculated as lactone, the initial specific rotation was as follows:

$$[\alpha]_D^{29} = \frac{+0.44° \times 100}{2 \times 0.647} = +34.0° \text{ (in water)}$$

The course of the hydrolysis of the lactone in aqueous solution was studied polarimetrically. The results obtained when $l = 2$ dm. and $c = 2.3$ are given in Table II.

The lactone would, therefore, appear to belong to "the moderately rapidly hydrolyzing"[10] group of δ-lactones since the time required for attainment of half the equilibrium value was estimated to be 14 hours.

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[10] Carter, S. R., Haworth, W. N., and Robinson, R. A., *J. Chem. Soc.*, 2125 (1930).

Simultaneous Hydrolysis and Oxidation of 2, 3, 4-Trimethyl Methylriboside with Nitric Acid—2 gm. of syrupy distilled trimethyl methylriboside were dissolved in 20 cc. of concentrated nitric acid (sp. gr. 1.42) at 20° and the temperature raised to 60°. At 65° a vigorous reaction took place and the temperature was maintained at 65–70° for 15 minutes. Afterwards the temperature was gradually raised to 95° and maintained there for 7 hours. The reaction mixture was cooled, diluted with distilled water, and evaporated under diminished pressure at 45° with continual additions of water. This procedure was repeated many times until the final distillate was free from nitric acid (2 days). The colorless viscid product was dried and then esterified by boiling it for 7 hours with 50 cc. of methyl alcohol containing 2 per cent of hydrogen chloride. After the mixture had been cooled, and the acid neutralized with dry silver carbonate, the methyl alcohol was removed by evaporation under diminished pressure and the product was distilled at high vacuum. A colorless very mobile liquid was collected at 77–78° at 0.02 mm. (bath temperature, 92–96°). Yield, 1.36 gm. $N_D^{24.5} = 1.4335$.

TABLE II

Conversion of Lactone into Acid

Time	$[\alpha]_D^{25}$	Lactone	Time	$[\alpha]_D^{25}$	Lactone
hrs.	*degrees*	*per cent*	*hrs.*	*degrees*	*per cent*
0	−4.4	100.0	67	+12.7	55.5
20	+8.4	66.7	115	+14.9	49.7
27	+9.7	63.3	139	+15.6	47.9
44	+10.5	61.2	163	+16.3	46.1
52	+11.9	57.6	191	+17.1	44.0
			241	+17.1	constant

Its composition was as follows:

3.035 mg. substance: 5.335 mg. CO_2 and 1.975 mg. H_2O.
3.241 " " : 15.390 " AgI.
$C_{10}H_{18}O_7$. Calculated. C 47.98, H 7.3, OMe 62.02
Found. " 47.94, " 7.3, " 62.68

It was optically inactive in water under conditions ($c = 1.2$, $l = 2$) where a specific rotation of ±1° would easily have been detectable. The analytical figures and the optical inactivity showed it to be the dimethyl ester of an inactive trimethoxyglutaric acid.

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THE ACTION OF BENZOIC PERACID ON SUBSTITUTED GLUCALS. II

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(Received for publication, June 29, 1931)

In Paper I of this series it was shown[1] that perbenzoic acid combines with triacetylglucal to yield a benzoyl triacetylglucose, but the question of the position of the benzoyl group was left undecided. Simultaneously Tanaka[2] described similar experiments, and further, he acetylated the benzoyl triacetylglucose, obtaining a benzoyl tetracetylglucose. We wish to point out that his product is identical with that obtained by Zemplén and László,[3] in 1915, by the action of silver benzoate upon bromotetracetylglucose. There thus remains little doubt but that in this compound the benzoyl group is situated at position (1) of the sugar chain.

In a similar way it has now been shown that the isolable product obtained by the action of perbenzoic acid on triacetylgalactal is 1-benzoyl-3, 4, 6-triacetylgalactose, that is, that when position (3) is acetylated, the only recognized product is a derivative of galactose. On the other hand, by the action of the same reagent, the non-substituted galactal forms talose predominatingly.

On comparison of the three examples so far studied of transformation of a simple "glucal" to a sugar, it is noted that a directive influence is apparently exercised by the position of the hydroxyl on carbon atom (3). Thus, from figures I and II and from figures III and IV it can be seen that both in glucal and in galactal the hydroxyl of carbon atom (3) is situated to the *left*, and the two glucals add the hydroxyl on carbon atom (2) to the *left* likewise,

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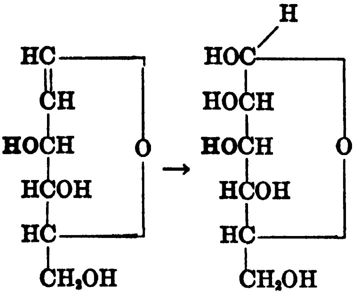
[1] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **88**, 513 (1930).

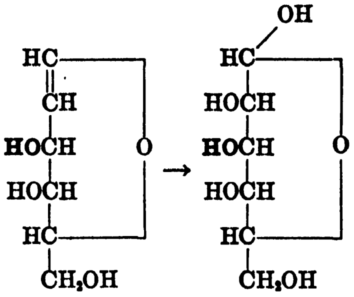
[2] Tanaka, C., *Bull. Chem. Soc. Japan*, **5**, 214 (1930).

[3] Zemplén, G., and László, E. D., *Ber. chem. Ges.*, **48**, 915 (1915).



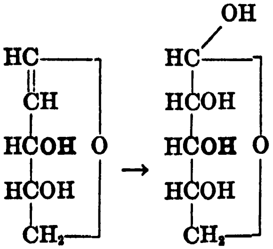
d-Glucal I

β-d-Mannose II



d-Galactal III

α-d-Talose IV



d-Arabinal V

α-d-Ribose VI

whereas in arabinal (V) the hydroxyl of carbon atom (3) is situated to the *right* and the "glucal" adds a hydroxyl on carbon atom (2) to the *right* likewise. In all three cases hydroxyls (2) and (3) of the preponderating sugar produced are in the *cis* position. Is it not possible that a transitory oxide is formed between carbon atoms (2) and (3), whereas when the hydroxyl of position (3) is blocked, the oxide forms between carbon atoms (1) and (2)?

It must be emphasized that, by the action of perbenzoic acid on glucals, *both* epimeric sugars result but that one largely predominates. The most striking example is the case of galactal. One epimer, however, predominates to such an extent that from the practical view-point the other may be disregarded. A review of the yields recorded in the literature substantiates this statement.

Glucal → 68 to 81 per cent yield of mannose.[4]
" → 60 " " " " α-methylmannoside.[5]
Cellobial → 90 " " " " 4-glucosido-α-mannose.[5,6]
" → 69 " " " " 4-glucosido-α-methylmannoside.[6]
Lactal → 70 " " " " 4-galactosido-α-mannose.[7]
d-Arabinal → a preponderance of *d*-ribose.[8]

On the other hand, when position (3) of the glucal is methylated or acetylated, the *isolable* product has *trans* groupings at positions (2) and (3). For example:

3-Methylglucal → 29.8; 27.5; 46.7 per cent yield of 3-methylglucose actually isolated.[1]

Triacetylglucal → 30.5; 22.1 per cent of benzoyl triacetylglucose actually isolated[1,2] and about 20 per cent of a mannose derivative.[9]

Trimethylglucal → a preponderance of trimethylglucose.[9]

On the basis of these observations it is possible, then, to predict that idose (idal) will lead to gulose, and that gulose (triacetyl gulal) will lead to idose; altrose (altral) will lead to allose, and that allose (triacetyl allal) will lead to altrose; xylose (xylal) will lead to lyxose, and that lyxose (diacetyl lyxal) will lead to xylose. Thus a way is suggested for the preparation and interconversion of the rarer sugars.

EXPERIMENTAL

Preparation of Crystalline Triacetylgalactal—120 gm. of zinc dust were added to 1000 cc. of cold 50 per cent aqueous acetic acid with vigorous mechanical stirring at 0°. Finely powdered bromoacetylgalactose (m.p., 81°; $[\alpha]_D^{24}$ = + 235.4° in benzene) was now added in ten portions of 10 gm. each, during 45 minutes. After all had been added, the reaction mixture was stirred for a further 100 minutes, the temperature being maintained at 0°, after which the mixture was filtered.

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[4] Bergmann, M., and Schotte, H., *Ber. chem. Ges.*, **54**, 440 (1921).

[5] Bergmann, M., and Schotte, H., *Ber. chem. Ges.*, **54**, 1564 (1921).

[6] Haworth, W. N., Hirst, E. L., Streight, H. R. L., Thomas, H. A., and Webb, J. I., *J. Chem. Soc.*, 2636 (1930).

[7] Haworth, W. N., Hirst, E. L., Plant, M. M. T., and Reynolds, R. J. W., *J. Chem. Soc.*, 2644 (1930). Bergmann, M., *Ann. Chem.*, **434**, 79 (1923). Watters, A. J., and Hudson, C. S., *J. Am. Chem. Soc.* **52**, 3472 (1930).

[8] Gehrke, M., and Aichner, F. X., *Ber. chem. Ges.*, **60**, 918 (1927).

[9] Hirst, E. L., and Woolvin, C. S., *J. Chem. Soc.*, 1131 (1931).

The filtrate was extracted ten times with 200 cc. portions of toluene, the toluene extract dried over anhydrous sodium sulfate and then evaporated to a thick syrup under diminished pressure at 40°. Two further portions of 200 cc. of toluene were run in and evaporated off. Finally, this syrup was dissolved in 200 cc. of benzene and evaporated to a thick syrup, from which traces of the solvent were removed at high vacuum at 40°. Yield, 58 gm.

As crystallization of this crude product could not be induced it was subjected to distillation at high vacuum. The main fraction (42 gm.) boiled at 134° at 0.01 mm. On cooling it was obtained as a colorless, fairly mobile syrup which crystallized spontaneously after standing at room temperature for 14 days, with occasional scratching with a glass rod. It was recrystallized from a mixture of ether and petroleum ether, crystals 2 cm. in length being readily obtained. The crystals melt at 30° and have a bitter taste. The product had the following composition.

3.465 mg. substance: 6.700 mg. CO_2 and 1.860 mg. H_2O.

$C_{12}H_{16}O_7$.	Calculated.	C 52.92, H 5.9
	Found.	" 52.74, " 6.0

Its rotation was

$$[\alpha]_D^{22} = \frac{-0.62^\circ \times 100}{2 \times 2.506} = -12.4^\circ \text{ (in chloroform)}$$

Action of Perbenzoic Acid on Triacetylgalactal—Perbenzoic acid was prepared in good yield by a slight modification of the method of Hibbert and Burt,[10] a solution of sodium methylate in methyl alcohol being found preferable to the ethyl derivative. 25 gm. of crystalline triacetylgalactal were dissolved in 50 cc. of dry chloroform and the resultant solution cooled to 0°. A cold solution of 13.97 gm. of perbenzoic acid in 270 cc. of dry chloroform was now added and the mixture kept at 8–10° for 48 hours and then at room temperature for 12 hours. At the end of this time a little more than the theoretical amount of perbenzoic acid had disappeared. The solution was evaporated to dryness under diminished pressure at 25° and extracted repeatedly with boiling petroleum ether (60–70°) under a reflux. The residual insoluble syrup

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[10] Hibbert, H., and Burt, C. P., *J. Am. Chem. Soc.*, 47, 2240 (1925).

(weight, 36 gm.) was dissolved in absolute ethyl alcohol and evaporated to a syrup which crystallized to a solid mass. It was recrystallized from 95 per cent ethyl alcohol. Yield, 12.5 gm. (33.2 per cent of the theoretical yield); m.p., 189°. Its analysis was as follows.

4.660 mg. substance: 9.545 mg. CO_2 and 2.240 mg. H_2O.
$C_{19}H_{22}O_{10}$. Calculated. C 55.58, H 5.4
Found. " 55.85, " 5.4

Its rotation was

$$[\alpha]_D^{22} = \frac{+0.55^\circ \times 100}{2 \times 1.145} = +24.0^\circ \text{ (in chloroform)}$$

The remaining 23.5 gm. of syrupy product could not be induced to crystallize. It consisted presumably of a mixture of 1-benzoyl triacetylgalactose with the corresponding talose derivative.

Acetylation of 1-Benzoyl-3, 4, 6-Triacetylgalactose—1.0 gm. of benzoyl triacetylgalactose was dissolved in a cold mixture of 10 cc. of pyridine and 10 cc. of acetic anhydride and the resulting solution was kept at 0° for 50 hours.

The reaction mixture was diluted with 200 cc. of chloroform and the solution washed successively with ice-cold dilute sulfuric acid, ice-cold dilute sodium bicarbonate solution, and finally with ice water until neutral. It was dried over anhydrous sodium sulfate and evaporated to a syrup. The product was dissolved in absolute alcohol and again evaporated, whereupon it crystallized spontaneously. Yield, 0.95 gm. On recrystallization from 95 per cent ethyl alcohol it had a melting point of 122°. Its composition was as follows:

4.342 mg. substance: 8.900 mg. CO_2 and 2.135 mg. H_2O.
$C_{21}H_{24}O_{11}$. Calculated. C 55.73, H 5.3
Found. " 55.89, " 5.5

Its rotation was

$$[\alpha]_D^{22} = \frac{-0.08^\circ \times 100}{2 \times 1.344} = -2.98^\circ \text{ (in chloroform)}$$

A mixed melting point determination with an authentic specimen of 1-benzoyl-2, 3, 4, 6-tetracetylgalactose (see next paragraph) showed no depression.

Action of Silver Benzoate upon Bromoacetylgalactose—To a solution of 10 gm. of crystalline bromoacetylgalactose in 50 cc. of toluene were added 7 gm. of dry, finely powdered silver benzoate and the mixture was heated under a reflux at 95° for 20 minutes. It was then shaken with a little charcoal, filtered, and the filtrate (having been found free of bromine) evaporated under diminished pressure at 35° to a very thick syrup. Final traces of solvent were removed at the high vacuum pump at 40°, whereupon the product crystallized immediately to a solid mass. Yield, 10.5 gm. After recrystallization from 95 per cent ethyl alcohol it had a melting point of 121° and its composition was as follows:

5.105 mg. substance: 10.390 mg. CO_2 and 2.435 mg. H_2O.
$C_{21}H_{24}O_{11}$. Calculated. C 55.73, H 5.3
Found. " 55.51, " 5.3

Its rotation was

$$[\alpha]_D^{23} = \frac{\pm 0.01° \times 100}{2 \times 3.420} = \pm 0.15° \text{ (in chloroform)}$$

Preparation of Crystalline Galactal (Talal)—To a solution of 70 gm. of barium hydroxide octahydrate in 800 cc. of distilled water were added 30 gm. of crystalline triacetylgalactal and the mixture was shaken on a shaking machine for 15 hours. The excess baryta was now neutralized by passing in gaseous carbon dioxide and the mixture filtered. The filtrate was shaken with a little charcoal, filtered, and evaporated to dryness under diminished pressure at 40°. The last traces of water were removed by running in two portions of absolute alcohol and evaporating to dryness each time. The mixture of barium acetate and galactal was now extracted three times with boiling absolute alcohol under a reflux, the extract shaken with a little charcoal, filtered, and evaporated under diminished pressure at 30°, the galactal crystallizing towards the end of the distillation. In order to remove traces of ash, the product was dissolved in 300 cc. of absolute alcohol, and 1700 cc. of dry ether were cautiously added with stirring. The flocculent precipitate was removed by filtration on a fluted filter and the resulting clear, colorless filtrate evaporated to dryness under diminished pressure. The galactal was recrystallized from 100 cc. of hot, dry, neutral ethyl acetate, being obtained in rosettes of fine needles on cooling slowly. Yield, 12 gm.; m.p., 100°. It had the following composition:

5.002 mg. substance: 9.042 mg. CO_2 and 3.102 mg. H_2O.

$C_6H_{10}O_4$.	Calculated.	C 49.28, H 6.9
	Found.	" 49.29, " 6.9

Galactal is intensely sweet, followed by a bitter after-taste. It is not hygroscopic when ash-free and dry. It readily decolorizes dilute bromine water. It does not give a positive test with Schiff's reagent and does not reduce boiling Fehling's solution. It is extremely sensitive to concentrated acids giving a dark brown precipitate, but is quite stable in alkaline solution. It is readily soluble in water, absolute alcohol, methyl alcohol, and warm ethyl acetate; much less soluble in ether and almost insoluble in benzene and petroleum ether. The presence of a slight trace of ash lowers the melting point considerably.

For obtaining galactal, a much less tedious method (a modification of that of Isbell[11]) was later used, the product being obtained in almost quantitative yield.

40 gm. of crystalline triacetylgalactal were dissolved in 1 liter of absolute methyl alcohol and the solution cooled to 0°. 30 cc. of a 0.25 molal solution of barium methylate in absolute methyl alcohol were added and the solution kept at 0° for 24 hours.

The solution was then saturated in the cold with carbon dioxide and 200 cc. of distilled water were added. The solution was again saturated with carbon dioxide and was then heated until gently boiling. A little charcoal was added, the mixture filtered, and the filtrate evaporated to dryness under diminished pressure at 30°. The crystalline product was dissolved in absolute ethyl alcohol and was again evaporated to dryness in order to remove traces of water.

The galactal was freed from traces of ash as previously indicated, and was then recrystallized from dry ethyl acetate. Yield, 21 gm.; m.p., 100°. Glucal is readily obtained crystalline by hydrolyzing triacetylglucal in the same manner.

Action of Perbenzoic Acid upon Galactal (Talal)—A solution of 7 gm. of galactal in 70 cc. of distilled water was cooled until ice formed, and then a cold solution of 7.7 gm. of perbenzoic acid in

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[11] Isbell, H. S., *Bureau Standards J. Research*, **5**, 1179 (1930).

98 cc. of ethyl acetate was added. The mixture was shaken at 8–10° for 2 hours, after which the ethyl acetate layer still contained perbenzoic acid, but the aqueous layer had lost its power of decolorizing very dilute bromine water. The two layers were separated, the ethyl acetate layer being twice shaken with a little water, and these aqueous extracts added to the main aqueous solution. The combined aqueous solution was now extracted three times with benzene, or until the benzene extract no longer gave a pink color with acidified potassium iodide solution, and then traces of benzene were removed under diminished pressure. It was diluted to 250 cc. and the aldohexose content of aliquot portions determined by the method of Willstätter and Schudel[12] as modified by Goebel[13] (see Table I).

2.5 cc. of sugar solution consumed 8.95 cc. 0.1 N iodine solution.

∴ yield of aldohexose = 8.06 gm. (93.4 per cent of theory). The specific rotation of this solution, calculated from the above aldohexose concentration, was

$$[\alpha]_D^{24} = \frac{+2.71^\circ \times 100}{2 \times 3.224} = +42.0^\circ \text{ (in water)}$$

This solution was now evaporated to a thick syrup under diminished pressure at room temperature. It was dissolved in absolute ethyl alcohol and evaporated to a thick syrup, the process being repeated several times more with ethyl alcohol and benzene. It was dissolved in ethyl alcohol and allowed to stand overnight at room temperature. The crystalline powder which had separated was filtered off (Fraction 1).

A second fraction (Fraction 2) was next obtained and on concentrating the mother liquor to a thick syrup in a vacuum desiccator over phosphorus pentoxide, and then triturating with methyl alcohol, this also crystallized spontaneously (Fraction 3).

Fraction 1—M.p. crude, 160°; weight, 0.26 gm. It had the following composition.

5.125 mg. substance: 7.535 mg. CO_2 and 3.035 mg. H_2O.

$C_6H_{12}O_6$.	Calculated.	C 39.98, H 6.7
	Found.	" 40.09, " 6.6

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[12] Willstätter, R., and Schudel, G., *Ber. chem. Ges.*, **51**, 780 (1918).

[13] Goebel, W. F., *J. Biol. Chem.*, **72**, 801 (1927).

Its rotation in water was

$$[\alpha]_D^{23} = \frac{+0.89° \times 100}{1 \times 1.104} = +80.6° \text{ (final)}$$

Fraction 2—M.p., 146°; weight, 0.41 gm. Its rotation in water was

$$[\alpha]_D^{21} = \frac{+1.43° \times 100}{1 \times 1.092} = +131.0° \text{ (initial)}$$

$$[\alpha]_D^{21} = \frac{+0.75° \times 100}{1 \times 1.092} = +68.7° \text{ (final)}$$

Fraction 3—M.p., 129–130°; sweet taste; weight, 3.12 gm. It had the following composition.

4.971 mg. substance: 7.417 mg. CO_2 and 3.045 mg. H_2O.
$C_6H_{12}O_6$. Calculated. C 39.98, H 6.7
Found. " 40.69, " 6.9
0.0648 gm. consumed 7.12 cc. 0.1 N iodine.
$C_5H_{11}O_5 \cdot CHO$. Calculated, 7.20 cc. 0.1 N iodine

Its rotation in water was

$$[\alpha]_D^{21} = \frac{+0.66° \times 100}{1 \times 1.052} = +62.7° \text{ (initial)}$$

$$[\alpha]_D^{21} = \frac{+0.34° \times 100}{1 \times 1.052} = +32.3° \text{ (final)}$$

Preparation of Various Substituted Phenylhydrazones

Fraction 1—To 0.2014 gm. of crystalline material in a small evaporating dish was added a solution of 0.2091 gm. of recrystallized *p*-bromophenylhydrazine dissolved in about 20 cc. of absolute methyl alcohol. The mixture was warmed gently until all the sugar had dissolved and was then evaporated to dryness. On being scratched with a glass rod, it crystallized to a solid mass. It was recrystallized from 5 per cent aqueous ethyl alcohol, being obtained as colorless feathery needles of m.p. 168–170. After a second recrystallization from 5 per cent ethyl alcohol, the melting point was unchanged. A mixed melting point determination with authentic galactose *p*-bromophenylhydrazone (m.p., 168–169°) gave m.p., 168–169°. Its composition was as follows:

3.800 mg. substance: 5.790 mg. CO_2 and 1.695 mg. H_2O.
4.670 " " : 0.320 cc. N_2 (756 mm. at 23.5°).
4.678 " " : 2.512 mg. AgBr.

$C_{12}H_{17}O_5N_2Br$.	Calculated.	C 41.25, H 4.9, N 8.03, Br 22.89
	Found.	" 41.55, " 5.0, " 7.85, " 22.85

Fraction 3—An attempt was made to prepare the *p-bromophenylhydrazone* under the conditions outlined above. After evaporation to dryness and trituration with a little methyl alcohol, the substance crystallized. After trituration with 5 per cent ethyl alcohol and filtration, the crude, dry product had a melting point of 146–148°. On being dissolved in 10 cc. of 5 per cent ethyl alcohol it would not crystallize on cooling, or nucleation. It was therefore evaporated slowly in a vacuum desiccator over phosphorus pentoxide, whereupon it crystallized completely, but was not further investigated on account of its great solubility.

Methylphenylhydrazone—0.5000 gm. of crystalline substance (Fraction 3) was treated with a solution of 0.3388 gm. of methylphenylhydrazine in 20 cc. of methyl alcohol. The solution was evaporated to dryness whereupon it crystallized to a solid mass. The crude product had a melting point of 146°. After one recrystallization from methyl alcohol, the melting point was 152°; after a second recrystallization from methyl alcohol it was 154°. The mixed melting point determination with authentic galactose methylphenylhydrazone (m.p., 185–186°) was 165–170°. The substance had the following composition.

4.237 mg. substance: 8.505 mg. CO_2 and 2.730 mg. H_2O.
4.065 " " : 0.352 cc. N_2 (756 mm. at 26.0°).

$C_{13}H_{20}O_5N_2$.	Calculated.	C 54.90, H 7.1, N 9.86
	Found.	" 54.75, " 7.2, " 9.83

Blanksma and van Ekenstein[14] found that talose methylphenylhydrazone had a melting point of 154° whereas von Braun and Bayer[15] give its melting point as 220–222° and in addition the latter investigators state that the *p*-bromophenylhydrazone separates quantitatively from 5 per cent alcoholic solution and has a melting point of 205°.

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[14] Blanksma, J. J., and van Ekenstein, W. A., *Chem. Weekblad*, 5, 771 (1908).

[15] von Braun, J., and Bayer, O., *Ber. chem. Ges.*, 58, 2215 (1925).

Oxidation with Nitric Acid

0.250 gm. of the crystalline sugar (Fraction 1) was dissolved in 5 cc. of 50 per cent nitric acid (by volume) and the solution kept at room temperature overnight. The solution turned pale green in color and crystals started to separate out. After standing for several days the nitric acid was removed by repeated evaporation with water. The crystalline product was triturated with dry acetone, dried, and weighed. Yield of mucic acid, 0.184 gm.; m.p., 216° with frothing and decomposition.

TABLE I

Yields of Aldohexose in Various Experiments

Amount of galactal used	0.1 N iodine consumed by 2.5 cc. of aldohexose solution	Total volume of aldohexose solution	Yield of aldohexose	α_D, 2 dm. tube	$[\alpha]_D$
gm.	*cc.*	*cc.*	*per cent*	*degrees*	*degrees*
7.0	8 95	250	93.4	+2.71	+42.0
3.0	9.75	100	94.9	+2.96	+42.2
6.8	9.14	250	98.1	+3.06	+46.5
5.0	5.99	250	87.4	+1.80	+42.0
5.0	6.05	250	88.5	+1.89	+43.4
5.0	5.99	250	87.5	+1.92	+44.6

Under similar conditions no crystalline material was isolated from the "talose" (Fraction 3).

Partial Purification of Talose

From these experiments it appears that Fraction 1 consisted of practically pure galactose, whereas Fraction 3 undoubtedly contained a large quantity of a less dextrorotatory sugar.

In further preparative experiments, details of which are recorded in Table I, the product obtained by the action of perbenzoic acid was isolated as a thick syrup (after removal of traces of perbenzoic acid by extraction with benzene) and after evaporation once with absolute ethyl alcohol the "talose" fraction (m.p., 129°) separated first, without recourse being had to nucleation. That this was not

TABLE II

Purification of Crude Talose

Weight of crude talose, m.p. 129°	Experiment 1 2.71 gm.	Experiment 2 3.50 gm.
Extraction with 80 per cent alcohol Extract 1; overnight at 25°	7 cc.; α_D^{25} = +9.12° (initial)	10 cc.; α_D^{25} = +7.79° (initial) α_D^{25} = +7.52° (final)
Extract 2; 5 min. at 25°	10 cc.; α_D^{25} = +4.90° (final)	10 cc.; α_D^{25} = +7.10° (initial) α_D^{25} = +5.70° (final)
Extract 3; 5 min. at 25°	10 cc.; α_D^{25} = +3.42° (final)	10 cc.; α_D^{25} = +4.77° (initial) α_D^{25} = +3.96° (final)
Yields A. Insoluble residue	0.33 gm. (m.p., 162–163°)	0.45 gm. (m.p., 169–170°)
B. Soluble, crystalline material from extracts (*a*) Containing no ammonia	1.72 gm.	1.92 gm.
(*b*) Containing ammonia (Experiments 1 and 2 combined)	1.78 gm.	

a pure specimen of talose, but contained traces of galactose, was revealed by extraction with 80 per cent ethyl alcohol (by volume). The details of two typical extraction experiments are given in Table II. The rotations were measured in a 2 dm. tube, the initial reading being made immediately after filtering a portion of the supernatant liquor, and the final reading being observed after addition of one drop of concentrated ammonia.

The insoluble sugar residue from Experiment 1 proved to be almost pure α-galactose[16] since its rotation in 80 per cent ethyl alcohol was

$$[\alpha]_D^{25} = \frac{+0.28^\circ \times 100}{2 \times 0.112} = +125.0^\circ \text{ (initial)}$$

$$[\alpha]_D^{25} = \frac{+0.17^\circ \times 100}{2 \times 0.112} = +75.9^\circ \text{ (final)}$$

The crude "talose" (m.p., 129°) used as starting material in Experiment 2 had the following rotation in 80 per cent ethyl alcohol.

$$[\alpha]_D^{25} = \frac{+0.17^\circ \times 100}{2 \times 0.108} = +78.7^\circ \text{ (initial)}$$

$$[\alpha]_D^{25} = \frac{+0.10^\circ \times 100}{2 \times 0.108} = +46.3^\circ \text{ (final)}$$

The crystalline ammonia-free product from these two experiments was combined and this once purified talose (3.64 gm.) submitted to a second purification in the same manner. It was first shaken for 2 hours with 10 cc. of 80 per cent alcohol at 27° and the filtrate evaporated slowly to give a crystalline powder (1.52 gm.). The insoluble sugar from the first extraction was then extracted for 5 minutes with 10 cc. of 80 per cent alcohol at 27°. On evaporation, the second extract gave 1.09 gm. of beautifully crystalline material. The insoluble residue, after drying, weighed 0.83 gm. It had a melting point of 130–135° and its rotation in water was

$$[\alpha]_D^{27} = \frac{+0.66^\circ \times 100}{2 \times 1.140} = +29.0^\circ \text{ (initial)}$$

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[16] Hudson, C. S., and Yanovsky, E., *J. Am. Chem. Soc.*, **39**, 1013 (1917).

$$[\alpha]_{\mathrm{D}}^{27} = \frac{+0.45^\circ \times 100}{2 \times 1.140} = +19.7^\circ \text{ (final)}$$

Since the solubility of α-galactose[16] in 80 per cent alcohol is 0.27 gm. per 100 cc. of solution (initial) and 0.65 gm. per 100 cc. of solution (final), it seems that talose is probably at least 40 times more soluble initially than α-galactose in 80 per cent alcohol.

At this time we cannot make a final statement of the properties of talose, but work on the preparation and purification of the substance is in progress. 2-Desoxygalactose has also been prepared. Its properties will be reported later.

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CHEMICAL STRUCTURE AND OPTICAL ROTATION

I. The Configurational Relationship of Disubstituted Propionic Acids Containing a Phenyl Group

II. On the Optically Active Trisubstituted Methanes Containing a Phenyl Group

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This paper deals in a general way with the effect on rotation of the phenyl group attached directly to the asymmetric carbon atom. The *first* object of the investigation was the correlation of the disubstituted propionic acids containing a phenyl group; the *second*, the correlation of the above series with the corresponding series of disubstituted acetic acids; the *third*, the correlation of the optically active hydrocarbons of the series of trisubstituted methane containing a phenyl group. The observations on the substances of the last group should serve as a check for the conclusions reached regarding the configurational relationship of the substituted acetic and propionic acids.

A. *Correlation of 3-Methylphenyl-, 3-Ethylphenyl-, and 3-Propylphenylpropionic Acids*

The configurations of these three substances were correlated by the method employed for the correlation of the configurations of the disubstituted propionic acids previously described.[1] The method of correlation is based on the evident requirement that configurationally related methylphenyl- and ethylphenylpropionic acids should lead to methylethylphenylmethanes of opposite rotations.

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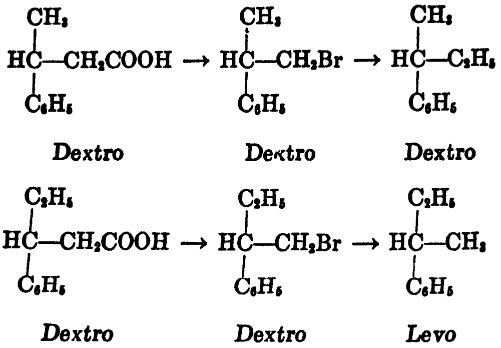
[1] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, 91, 77, 687 (1931).



$$\underset{\mathrm{C_6H_5}}{\overset{\mathrm{CH_3}}{\mathrm{HC}}}\mathrm{-CH_2COOH} \rightarrow \underset{\mathrm{C_6H_5}}{\overset{\mathrm{CH_3}}{\mathrm{HC}}}\mathrm{-CH_2Br} \rightarrow \underset{\mathrm{C_6H_5}}{\overset{\mathrm{CH_3}}{\mathrm{HC}}}\mathrm{-C_2H_5}$$

Dextro Dextro Dextro

$$\underset{\mathrm{C_6H_5}}{\overset{\mathrm{C_2H_5}}{\mathrm{HC}}}\mathrm{-CH_2COOH} \rightarrow \underset{\mathrm{C_6H_5}}{\overset{\mathrm{C_2H_5}}{\mathrm{HC}}}\mathrm{-CH_2Br} \rightarrow \underset{\mathrm{C_6H_5}}{\overset{\mathrm{C_2H_5}}{\mathrm{HC}}}\mathrm{-CH_3}$$

Dextro *Dextro* *Levo*

TABLE I

Configurationally Related Compounds Derived from Phenylpropionic Acids

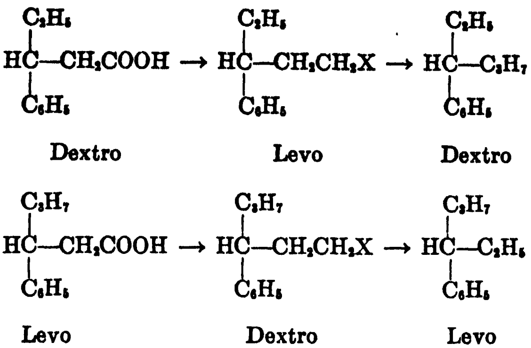
$[\mathrm{M}]_{\mathrm{D}}^{25}$*

	$\mathrm{-CH_2COOH}$	$\mathrm{-CH_2COOC_2H_5}$	$\mathrm{-CH_2CH_2OH}$	$\mathrm{-CH_2CH_2Br}$	$\mathrm{-CH_3}$	$\mathrm{-C_2H_5}$	$\mathrm{-C_3H_7}$ (*n*)
$\mathrm{CH_3}$ $\mathrm{HC-}$ $\mathrm{C_6H_5}$	+20.31	+14.23	+13.72	+39.45	0	+9.14	
$\mathrm{C_2H_5}$ $\mathrm{HC-}$ $\mathrm{C_6H_5}$	+31.64†	+15.27	+7.46	+50.46	−2.59	0	+0.91
$\mathrm{C_3H_7}$ (*n*) $\mathrm{HC-}$ $\mathrm{C_6H_5}$	−11.39	−7.60	−6.47	−26.42		−1.12	0

* All rotations are in homogeneous state unless specified.

† In benzene.

Likewise 3-ethylphenyl- and 3-propylphenylpropionic acids are configurationally related when the ethylpropylphenylmethanes derived from them rotate in opposite directions.



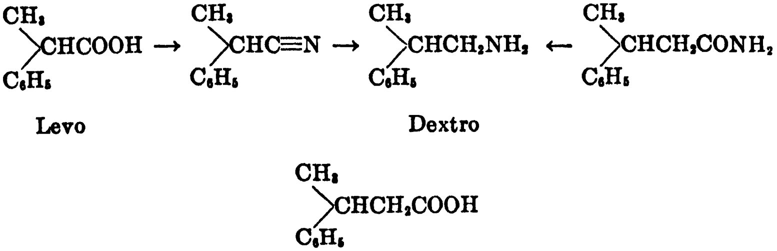
$$\begin{array}{ccccc} C_2H_5 & & C_2H_5 & & C_2H_5 \\ HC\text{—}CH_2COOH & \rightarrow & HC\text{—}CH_2CH_2X & \rightarrow & HC\text{—}C_3H_7 \\ C_6H_5 & & C_6H_5 & & C_6H_5 \\ \text{Dextro} & & \text{Levo} & & \text{Dextro} \\ \\ C_3H_7 & & C_3H_7 & & C_3H_7 \\ HC\text{—}CH_2COOH & \rightarrow & HC\text{—}CH_2CH_2X & \rightarrow & HC\text{—}C_2H_5 \\ C_6H_5 & & C_6H_5 & & C_6H_5 \\ \text{Levo} & & \text{Dextro} & & \text{Levo} \end{array}$$

The observations are summarized in Table I.

It can be seen from Table I that *dextro*-methyl and dextro-ethylphenyl- and *levo*-propylphenylacetic acids and also that dextro-1,3-methylphenyldextro-1,1-ethylphenylpropionic and *levo*-1,1-propylphenylpropionic acids are configurationally related.

B. Correlation of Configurations of Disubstituted Derivatives of Acetic and Propionic Acids

In order to correlate the configurations of 3-methylphenyl and propionic and methylphenylacetic acids, each is converted into 2-methylphenylethylamine (1).



Levo

Dextro

Dextro

The results obtained by this method are summarized in Table II.

From Table II it can be seen that configurationally related disubstituted acetic and propionic acids rotate in the opposite directions.

TABLE II

Relationship of Phenylacetic Acids to Phenylpropionic Acids

$[M]_D^{25}$

	$-COOH$	$-CH_2NH_2$	$-CH_2COOH$*
CH_3 $HC-$ C_6H_5	Levo†	+13.31	+1.56
C_2H_5 $HC-$ C_6H_5	Levo†	−4.86	+4.85
C_3H_7 (*n*) $HC-$ C_6H_5	Dextro†	Levo (HBr)	Levo

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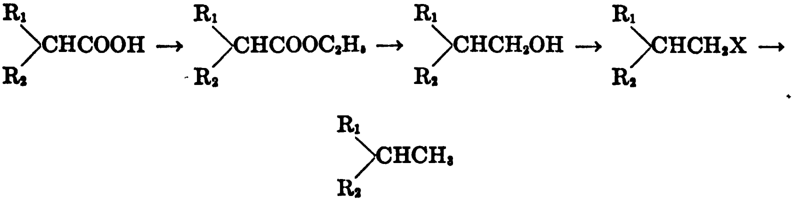
* See Table I for configurational relationship of these acids.

† Levene, P. A., Mikeska, L. A., and Passoth, K., *J. Biol. Chem.*, **88**, 30 (1930).

Configurational Relationships of Optically Active Hydrocarbons of the Series of Substituted Methanes Containing a Phenyl Group

The correlation of the configurations of the hydrocarbons of this series is based upon the method of their preparation. All hydrocarbons were prepared from the disubstituted acids by the following set of reactions.



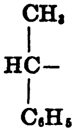
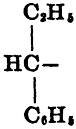
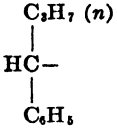
$$R_1R_2CHCOOH \rightarrow R_1R_2CHCOOC_2H_5 \rightarrow R_1R_2CHCH_2OH \rightarrow R_1R_2CHCH_2X \rightarrow R_1R_2CHCH_3$$

The hydrocarbons containing methyl, ethyl, and propyl groups were derived from the acids the configurations of which have been

TABLE III

Configurationally Related Phenyl Acids

$[M]_D^{25}$

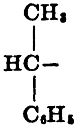
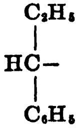
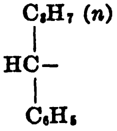
	$-COOH$	$-CH_2COOH$	$-CH_2CH_2CH_2COOH$
 $\begin{array}{l} CH_3 \\ \vert \\ HC- \\ \vert \\ C_6H_5 \end{array}$	Levo[*]	+20.31	+11.62
 $\begin{array}{l} C_2H_5 \\ \vert \\ HC- \\ \vert \\ C_6H_5 \end{array}$	Levo[*]	+31.64	+8.78
 $\begin{array}{l} C_3H_7\ (n) \\ \vert \\ HC- \\ \vert \\ C_6H_5 \end{array}$	Dextro[*]	−11.39	−1.66

[*] Levene, P. A., Mikeska, L. A., and Passoth, K., *J. Biol. Chem.*, **88**, 30 (1930).

TABLE IV

Configurationally Related Esters of Phenyl Acids

$[M]_D^{25}$

	$-COOC_2H_5$	$-CH_2COOC_2H_5$	$-CH_2CH_2CH_2COOC_2H_5$
 $\begin{array}{l} CH_3 \\ \vert \\ HC- \\ \vert \\ C_6H_5 \end{array}$	Levo[*]	+14.23	+9.21
 $\begin{array}{l} C_2H_5 \\ \vert \\ HC- \\ \vert \\ C_6H_5 \end{array}$	Levo[*]	+15.27	+4.95
 $\begin{array}{l} C_3H_7\ (n) \\ \vert \\ HC- \\ \vert \\ C_6H_5 \end{array}$	Dextro[*]	−7.60	−1.09

[*] Levene, P. A., Mikeska, L. A., and Passoth, K., *J. Biol. Chem.*, **88**, 30 (1931).

TABLE V

Configurationally Related Phenyl Carbinols

$[M]_D^{25}$

	$-CH_2OH$	$-CH_2CH_2OH$	$-CH_2CH_2CH_2OH$
$\begin{array}{l} CH_3 \\ \vert \\ HC- \\ \vert \\ C_6H_5 \end{array}$	Levo*	+13.72	+10.57
$\begin{array}{l} C_2H_5 \\ \vert \\ HC- \\ \vert \\ C_6H_5 \end{array}$	Levo*	+7.46	+4.67
$\begin{array}{l} C_3H_7\ (n) \\ \vert \\ HC- \\ \vert \\ C_6H_5 \end{array}$		−6.47	−2.09

* Levene, P. A., Mikeska, L. A., and Passoth, K., *J. Biol. Chem.*, **88**, 30 (1930).

TABLE VI

Configurationally Related Phenyl Halides

$[M]_D^{25}$

	$-CH_2Cl$	$-CH_2CH_2Br$	$-CH_2CH_2CH_2CH_2Br$
$\begin{array}{l} CH_3 \\ \vert \\ HC- \\ \vert \\ C_6H_5 \end{array}$	Dextro*	+39.45	+14.72
$\begin{array}{l} C_2H_5 \\ \vert \\ HC- \\ \vert \\ C_6H_5 \end{array}$	Levo*	+50.46	+12.56
$\begin{array}{l} C_3H_7\ (n) \\ \vert \\ HC- \\ \vert \\ C_6H_5 \end{array}$		−26.42	−5.39

* Levene, P. A., Mikeska, L. A., and Passoth, K., *J. Biol. Chem.*, **88**, 30 (1930).

discussed in the two preceding sections. The configurations of the hydrocarbons, the highest aliphatic radicle of which was a butyl, could be established by their synthesis from disubstituted 1,1-valeric acids. The latter, indeed, were prepared from the disubstituted propionic acids through the malonic ester synthesis. The intermediate derivatives leading to the lower hydrocarbons are given in Tables III to VI. Those leading to the hydrocarbons containing a butyl radicle are given in Table VII. The hydrocarbons are tabulated in Table VIII.

TABLE VII

Experimental Values of Configurationally Related Compounds in Synthesis of Hydrocarbons, Containing a Phenyl Group

$[M]_D^{25}$

	$—CH_2CH_2Br$	$—CH_2CH_2CH_2COOH$	$—CH_2CH_2CH_2COOC_2H_5$	$—CH_2CH_2CH_2CH_2OH$	$—CH_2CH_2CH_2CH_2Br$	$—CH_2CH_2CH_2CH_3$
CH_3 | HC— | C_6H_5	+12.84	+3.86	+3.06	+3.51	+4.89	+3.18
C_2H_5 | HC— | C_6H_5	+25.08	+4.37	+2.46	+2.82	+6.25	+1.71
$C_3H_7(n)$ | HC— | C_6H_5	−28.56	−1.80	−1.19	−2.27	−5.86	+1.18

The significant feature of Table VIII is the fact that it demonstrates the relationships between the direction of rotation and the allocation of the heavier aliphatic group. A rearrangement be-

TABLE VIII

Configurationally Related Phenyl Hydrocarbons. $[M]^{25}_{D}$

CH_3 HC—CH_2COOH C_6H_5	CH_3 HC—CH_3 C_6H_5	CH_3 HC—C_2H_5 C_6H_5	CH_3 HC—C_3H_7 (*n*) C_6H_5	CH_3 HC—C_4H_9 (*n*) C_6H_5
+ 20.31	0	+ 9.14		+ 9.86
C_2H_5 HC—CH_2COOH C_6H_5	C_2H_5 HC—CH_3 C_6H_5	C_2H_5 HC—C_2H_5 C_6H_5	C_2H_5 HC—C_3H_7 (*n*) C_6H_5	C_2H_5 HC—C_4H_9 (*n*) C_6H_5
+ 31.64*	Levo†	0	+ 0.91	+ 3.44
C_3H_7 (*n*) HC—CH_2COOH C_6H_5	C_3H_7 (*n*) HC—CH_3 C_6H_5	C_3H_7 (*n*) HC—C_2H_5 C_6H_5	C_3H_7 (*n*) HC—C_3H_7 (*n*) C_6H_5	C_3H_7 (*n*) HC—C_4H_9 (*n*) C_6H_5
− 11.39		− 1.12	0	+ 1.09

* In benzene.

† Relative values not known.

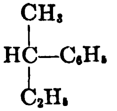
tween the relative positions of the heavier and lighter aliphatic groups leads to a change in rotation. This conduct is to be expected on the basis of the observations on the optically active hydrocarbons of the aliphatic series. Hence, there need be no doubt of the correctness of the relationship of the hydrocarbons as given in Table VIII. This fact in its turn gives confidence in the earlier conclusions as to the configurational relationship of the acids from which they are derived.

Summary of Results

The results of the major part of the observations discussed in the preceding section are summarized in Table IX.

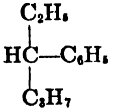
A mere glance at the structure and the rotations of these substances brings out certain general relationships between chemical structure and optical activity.

1. The rotations of the hydrocarbons demonstrate the effect of the respective positions of the lighter and heavier aliphatic groups on the direction of the rotation. Thus, presenting the substance in the form



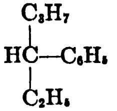
$$\begin{array}{l} CH_3 \\ | \\ HC-C_6H_5 \\ | \\ C_2H_5 \end{array}$$

Dextro



$$\begin{array}{l} C_2H_5 \\ | \\ HC-C_6H_5 \\ | \\ C_3H_7 \end{array}$$

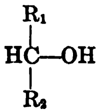
Dextro



$$\begin{array}{l} C_3H_7 \\ | \\ HC-C_6H_5 \\ | \\ C_2H_5 \end{array}$$

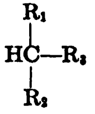
Levo

there appears a relationship which has previously been noted in the case of secondary carbinols of the type



$$\begin{array}{l} R_1 \\ | \\ HC-OH \\ | \\ R_2 \end{array}$$

and of the aliphatic hydrocarbons of the type



$$\begin{array}{l} R_1 \\ | \\ HC-R_3 \\ | \\ R_2 \end{array}$$

in which the position of the heavier group (R_2) determined the direction of rotation.

TABLE IX

Configurationally Related Compounds Containing a Phenyl Group. $[M]^{25}_D$

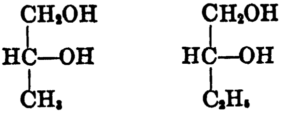
CH_3 HC— C_6H_5	CH_3	0	CH_3 HC— C_6H_5	C_2H_5	+ 9.14	CH_3 HC— C_6H_5	C_4H_9 (*n*)	+ 9 86
	CH_2Cl	+ 2 5		CH_2CH_2Br	+ 39.45		$CH_2CH_2CH_2CH_2Br$	+ 14 72
	CH_2OH	− 22.1*		CH_2CH_2OH	+ 13.72		$CH_2CH_2CH_2CH_2OH$	+ 10.57
	$COOC_2H_5$	Levo		$CH_2COOC_2H_5$	+ 14.23		$CH_2CH_2CH_2COOC_2H_5$	+ 9.21
	$COOH$	− 96.8		CH_2COOH	+ 20.31		$CH_2CH_2CH_2COOH$	+ 11.62
	CH_2NH_2	+ 23.9		CH_2NH_2	+ 1.56			
C_2H_5 HC— C_6H_5	CH_3	Levo	C_2H_5 HC— C_6H_5	C_2H_5	0	C_2H_5 HC— C_6H_5	C_4H_9 (*n*)	+ 3.44
	CH_2Br	− 17.8		CH_2CH_2Br	+ 50.46		$CH_2CH_2CH_2CH_2Br$	+ 12.56
	CH_2OH	− 28.3		CH_2CH_2OH	+ 7.46		$CH_2CH_2CH_2CH_2OH$	+ 4.67
	$COOC_2H_5$	− 139.3*		$CH_2COOC_2H_5$	+ 15.27		$CH_2CH_2CH_2COOC_2H_5$	+ 4.95
	$COOH$	− 139.4		CH_2COOH	+ 31.64		$CH_2CH_2CH_2COOH$	+ 8.78
	CH_2NH_2	− 6.8		CH_2NH_2	− 4.86			
C_3H_7 HC— C_6H_5	CH_3		C_3H_7 HC— C_6H_5	C_2H_5	− 1.12	C_3H_7 HC— C_6H_5	C_4H_9 (*n*)	+ 1.18
	CH_2Br			CH_2CH_2Br	− 26.42		$CH_2CH_2CH_2CH_2Br$	− 5.39
	CH_2OH			CH_2CH_2OH	− 6.47		$CH_2CH_2CH_2CH_2OH$	− 2.09
	$COOC_2H_5$	+ 40.4*		$CH_2COOC_2H_5$	− 7.60		$CH_2CH_2CH_2COOC_2H_5$	− 1.09
	$COOH$	+ 27.7*		CH_2COOH	− 11.39		$CH_2CH_2CH_2COOH$	− 1.66
	CH_2NH_2	− 5.2		CH_2NH_2	Levo			

* In ether.

2. The rotations of the configurationally related disubstituted acetic and propionic acids containing a phenyl group clearly show that the effect of a polar group or of a group absorbing in the near ultra-violet region differs with the distance of this group from the asymmetric carbon atom. These observations are in harmony with those of the earlier workers[2] on relationship between chemical structure and optical activity, as well as with the earlier observations made in this laboratory.[1]

3. The fact that configurationally related ethylphenyl- and propylphenylacetic acids rotate in opposite directions and that configurationally related ethylphenyl- and propylphenylpropionic acids likewise rotate in opposite directions need special consideration.

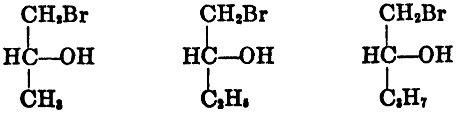
In a way, similar relationships have been observed in the aliphatic series, in substances containing two polar groups, as can be seen from the following figures.



$$\begin{array}{c} CH_2OH \\ | \\ HC{-}OH \\ | \\ CH_3 \end{array} \qquad \begin{array}{c} CH_2OH \\ | \\ HC{-}OH \\ | \\ C_2H_5 \end{array}$$

Levo Dextro

I

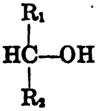


$$\begin{array}{c} CH_2Br \\ | \\ HC{-}OH \\ | \\ CH_3 \end{array} \qquad \begin{array}{c} CH_2Br \\ | \\ HC{-}OH \\ | \\ C_2H_5 \end{array} \qquad \begin{array}{c} CH_2Br \\ | \\ HC{-}OH \\ | \\ C_3H_7 \end{array}$$

Levo Levo Dextro

II

If the rotations of these substances are compared with those of simple secondary carbinols of the type



$$\begin{array}{c} R_1 \\ | \\ HC{-}OH \\ | \\ R_2 \end{array}$$

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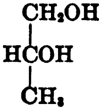
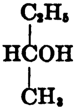
[2] Drude, P., Optiks, Leipsic (1900). Tschugaeff, L., *Tr. Faraday Soc.*, 10, 70 (1914). Kuhn, W., *Tr. Faraday Soc.*, 26, 293 (1930).

then it will have to be concluded that in



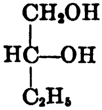
$$\begin{array}{c} CH_2OH \\ | \\ HCOH \\ | \\ CH_3 \end{array}$$

the group $-CH_2OH$ functions as the heavier group as in



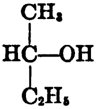
$$\begin{array}{c} C_2H_5 \\ | \\ HCOH \\ | \\ CH_3 \end{array}$$

whereas, in



$$\begin{array}{c} CH_2OH \\ | \\ HC-OH \\ | \\ C_2H_5 \end{array}$$

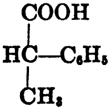
it functions as the lighter group as in



$$\begin{array}{c} CH_3 \\ | \\ HC-OH \\ | \\ C_2H_5 \end{array}$$

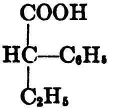
In the case of bromohydrins, the $-CH_2Br$ functions as the heavier group in the first two substances. That means it functions as a group heavier than methyl and ethyl but functions as a lighter group compared with propyl and butyl groups.

If, then, the substituted acetic and propionic acids are written in an analogous way; namely,



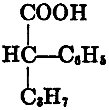
$$\begin{array}{c} COOH \\ | \\ HC-C_6H_5 \\ | \\ CH_3 \end{array}$$

Levo



$$\begin{array}{c} COOH \\ | \\ HC-C_6H_5 \\ | \\ C_2H_5 \end{array}$$

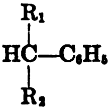
Levo



$$\begin{array}{c} COOH \\ | \\ HC-C_6H_5 \\ | \\ C_3H_7 \end{array}$$

Dextro

and if these substances are compared with the hydrocarbons of the type



$$\begin{array}{c} R_1 \\ | \\ HC-C_6H_5 \\ | \\ R_2 \end{array}$$

then it will appear that the group COOH functions as a heavier group than $-CH_3$ and $-C_2H_5$ and functions as a lighter group than $-C_3H_7$. The same is true in the case of disubstituted propionic and valeric acids; namely, $-CH_2COOH$ and $CH_2CH_2CH_2$-COOH functions as heavier groups than methyl and ethyl and lighter than propyl.

These considerations lead to still another important conclusion; namely, that the direction of rotation in the individual members of this group of substances is not always determined by the group which possesses an ultraviolet absorption band in the neighborhood of the visible spectrum.

EXPERIMENTAL

Levo-2-Phenylbutyric Acid (4)—The inactive acid was prepared from methylphenylbromomethane and ethyl malonate. 340 gm. of inactive acid were dissolved in 6 liters of hot acetone and 1600 gm. of quinine were added. The acetone solution was allowed to stand in a cold room until crystallization took place. After seven crystallizations the salt was decomposed by shaking with 10 per cent hydrochloric acid. The organic acid was extracted with ether, then fractionated. B.p., 134° at 4 mm.; yield, 55 gm.; $n_{D}^{25} = 1.5169$; $D\frac{26}{4} = 1.066$.

$$[\alpha]_D^{26} = \frac{-13.20°}{1 \times 1.066} = -12.38°. \qquad [M]_D^{26} = -20.31° \text{ (homogeneous)}$$

$$[\alpha]_D^{24} = \frac{-1.25° \times 100}{1 \times 0.5520 \times 20} = -11.32°. \quad [M]_D^{24} = -18.57° \text{ (in benzene)}$$

4.550 mg. substance: 12.180 mg. CO_2 and 3.090 mg. H_2O.
$C_{10}H_{12}O_2$. Calculated. C 73.17, H 7.37
Found. " 72.82, " 7.60

Levo-Ethyl Ester of 2-Phenylbutyric Acid (4)—110 gm. of 2-phenylbutyric acid (4), $[\alpha]_D^{26} = -12.39°$, were mixed with 250 cc. of absolute alcohol and 6 cc. of concentrated sulfuric acid. The mixture was heated for 1 hour on a steam bath. The excess alcohol was distilled off under reduced pressure and the residue extracted with ether. The ether solution was washed with water, dried with sodium sulfate, then fractionated. B.p., 111° at 4 mm.; yield, 116 gm.; $n_D^{25} = 1.4918$; $D\frac{25}{4} = 0.996$.

$$[\alpha]_D^{25} = \frac{-7.38°}{1 \times 0.996} = -7.41°. \quad [M]_D^{25} = -14.24° \text{ (homogeneous)}$$

3.540 mg. substance: 9.745 mg. CO_2 and 2.620 mg. H_2O.
$C_{12}H_{16}O_2$. Calculated. C 74.95, H 8.39
Found. " 75.06, " 8.28

Levo-3-Phenyl-1-Butanol—100 gm. of ethyl ester of 2-phenylbutyric acid (4), $[\alpha]_D^{25} = -7.41°$, were dissolved in 500 cc. of absolute alcohol and this solution dropped into a suspension of 150 gm. of sodium in 1 liter of boiling toluene with rapid stirring. Alcohol was then added to destroy the excess sodium. The reaction mixture was cooled. Ice was added and the carbinol extracted from the alkaline solution with ether. The ether solution was dried, then fractionated. B.p., 117° at 8 mm.; yield, 76 gm.; $n_D^{25} = 1.5201$; $D\frac{25}{4} = 0.986$.

$$[\alpha]_D^{25} = \frac{-9.02°}{1 \times 0.986} = -9.15°. \quad [M]_D^{25} = -13.74° \text{ (homogeneous)}$$

4.245 mg. substance: 12.465 mg. CO_2 and 3.600 mg. H_2O.
$C_{10}H_{14}O$. Calculated. C 79.94, H 9.40
Found. " 80.07, " 9.49

Levo-1-Bromo-3-Phenylbutane—12 gm. of 3-phenyl-1-butanol, $[\alpha]_D^{25} = -9.15°$, were cooled in ice and 15 gm. of phosphorus tribromide slowly added. The product was heated 1 hour on a steam bath, then cooled, and poured on ice. The oily layer was separated, shaken with strong hydrobromic acid solution, extracted with ether, dried, and then fractionated. B.p., 120° at 17 mm.; yield, 15 gm.; $n_D^{25} = 1.5371$; $D\frac{25}{4} = 1.314$.

$$[\alpha]_D^{25} = \frac{-24.33°}{1 \times 1.314} = -18.52°. \quad [M]_D^{25} = -39.44° \text{ (homogeneous)}$$

4.440 mg. substance: 9.190 mg. CO_2 and 2.395 mg. H_2O.
$C_{10}H_{13}Br$. Calculated. C 56.33, H 6.15
Found. " 56.44, " 6.03

Levo-Methylethylphenylmethane—A Grignard reagent was prepared from 6 gm. of magnesium in dry ether and 50 gm. of 1-

bromo-3-phenylbutane, $[\alpha]_D^{25} = -18.52°$. The Grignard reagent was poured on ice and dilute hydrochloric acid and the hydrocarbon extracted with ether. The ether was evaporated and the residue shaken with a small amount of ice-cold concentrated sulfuric acid, washed with sodium carbonate solution, then water, and dried over dry sodium sulfate. It was then distilled from a small piece of metallic sodium. B.p., 68° at 20 mm.; yield, 21 gm.; $D_4^{24} = 0.868$.

$$[\alpha]_D^{24} = \frac{-5.92°}{1 \times 0.868} = -6.82°. \quad [M]_D^{24} = -9.14° \text{ (homogeneous)}$$

4.693 mg. substance: 15.345 mg. CO_2 and 4.445 mg. H_2O.
$C_{10}H_{14}$. Calculated. C 89.48, H 10.52
Found. " 89.16, " 10.59

Levo-3-Phenylvaleric Acid (5)—The inactive acid was prepared from ethylphenylbromomethane and ethyl malonate. 356 gm. of the inactive acid were dissolved in 4 liters of hot acetone and 756 gm. of quinine added. The salt was crystallized by letting the solution stand overnight in a cold room. After eight crystallizations it was decomposed by 10 per cent hydrochloric acid and the organic acid recovered as described for 3-methylphenylpropionic acid. B.p., 140° at 4 mm.; yield, 53 gm. This material solidified at room temperature.

$$[\alpha]_D^{25} = \frac{-1.60° \times 100}{1 \times 20 \times 0.4851} = -16.5°. \quad [M]_D^{25} = -29.4° \text{ (in benzene)}$$

4.235 mg. substance: 11.570 mg. CO_2 and 3.015 mg. H_2O.
$C_{11}H_{14}O_2$. Calculated. C 74.11, H 7.92
Found. " 74.50, " 7.99

Levo-Ethyl Ester of 3-Phenylvaleric Acid (5)—100 gm. of 3-phenylvaleric acid (5), $[\alpha]_D^{25} = -16.48°$ (in benzene), were mixed with 250 cc. of absolute alcohol and 6 cc. of concentrated sulfuric acid. The esterification was carried out as described for ethyl ester of 3-methylphenylpropionic acid. B.p., 110° at 2 mm.; yield, 105 gm.

$$[\alpha]^{25}_{D} = \frac{-7.30^\circ}{1 \times 0.985} = -7.41^\circ. \quad [M]^{25}_{D} = -15.02^\circ \text{ (homogeneous)}$$

3.780 mg. substance: 10.480 mg. CO_2 and 3.075 mg. H_2O.
$C_{13}H_{18}O_2$. Calculated. C 75.68, H 8.80
Found. " 75.60, " 9.10

Levo-3-Phenyl-1-Pentanol—100 gm. of the ethyl ester of 3-phenylvaleric acid (5), $[\alpha]^{25}_{D} = -7.41^\circ$, were dissolved in 500 cc. of absolute alcohol. This solution was dropped into 140 gm. of finely divided sodium in 800 cc. of boiling toluene with stirring. The reduction was carried out as described for 3-phenyl-1-butanol. B.p., 108° at 1 mm.; yield, 65 gm.; $n^{25}_{D} = 1.5145$; $D^{25}_{4} = 0.967$.

$$[\alpha]^{25}_{D} = \frac{-4.38^\circ}{1 \times 0.967} = -4.53^\circ. \quad [M]^{25}_{D} = -7.43^\circ \text{ (homogeneous)}$$

4.050 mg. substance: 11.995 mg. CO_2 and 3.610 mg. H_2O.
$C_{11}H_{16}O$. Calculated. C 80.42, H 9.82
Found. " 80.76, " 9.97

Levo-1-Bromo-3-Phenylpentane—10 gm. of 3-phenyl-1-pentanol, $[\alpha]^{25}_{D} = -4.54^\circ$, were brominated by 15 gm. of phosphorus tribromide as described for 1-bromo-3-phenylbutane. B.p., 127° at 15 mm.; yield, 12 gm.; $n^{25}_{D} = 1.5287$; $D^{25}_{4} = 1.224$.

$$[\alpha]^{25}_{D} = \frac{-27.21^\circ}{1 \times 1.224} = -22.23^\circ. \quad [M]^{25}_{D} = -50.47^\circ \text{ (homogeneous)}$$

4.335 mg. substance: 9.225 mg. CO_2 and 2.620 mg. H_2O.
$C_{11}H_{15}Br$. Calculated. C 58.17, H 6.66
Found. " 58.03, " 6.76

Dextro-Ethyl-n-Propylphenylmethane—A Grignard reagent was prepared from 28 gm. of 1-bromo-3-phenylpentane, $[\alpha]^{25}_{D} = +16.53^\circ$, and 4 gm. of magnesium in dry ether. To this were added 5 gm. of paraformaldehyde; and the mixture was allowed to stand overnight. The Grignard reagent was decomposed in the usual way and the crude carbinol distilled. B.p., 115° at 3 to 4 mm.; yield, 13 gm. This was not purified further.

13 gm. of the carbinol were treated with 25 gm. of phosphorus tribromide as described for 1-bromo-3-phenylbutane. Yield, 12 gm.; b.p., 110° at 3 to 4 mm. A Grignard reagent was prepared from this halide and 1.5 gm. of finely divided magnesium in ether.

This was poured on ice and the hydrocarbon extracted and purified as described for methylethylphenylmethane. B.p., 103° at 25 mm.; yield, 3 gm.; $D\frac{24}{4} = 0.863$.

$$[\alpha]^{24}_{D} = \frac{+0.49^\circ}{1 \times 0.863} = +0.57^\circ. \quad [M]^{24}_{D} = +0.92^\circ \text{ (homogeneous)}$$

4.331 mg. substance: 13.994 mg. CO_2 and 4.250 mg. H_2O.
$C_{12}H_{18}$. Calculated. C 88.81, H 11.19
Found. " 88.11, " 10.98

Dextro-4-Phenylcaproic Acid (*6*)—The inactive acid was prepared from *n*-propylphenylbromomethane and ethyl malonate. 460 gm. of the inactive acid were dissolved in 6 liters of boiling acetone and 870 gm. of quinine were added. The solution was allowed to stand in the cold room overnight for crystallization. After six crystallizations the quinine salt was decomposed and the acid extracted as described for 3-methylphenylpropionic acid. B.p., 152° at 4 mm.; yield, 70 gm.; $n^{25}_{D} = 1.5078$; $D\frac{25}{4} = 1.025$.

$$[\alpha]^{25}_{D} = \frac{+6.08^\circ}{1 \times 1.025} = +5.93^\circ. \quad [M]^{25}_{D} = +11.39^\circ \text{ (homogeneous)}$$

4.395 mg. substance: 12.145 mg. CO_2 and 3.305 mg. H_2O.
$C_{12}H_{16}O_2$. Calculated. C 74.95, H 8.39
Found. " 75.35, " 8.41

Dextro-Ethyl Ester of 4-Phenylcaproic Acid (*6*)—75 gm. of 4-phenylcaproic acid (6), $[\alpha]^{25}_{D} = +5.93^\circ$, were mixed with 175 cc. of absolute alcohol and 4 cc. of sulfuric acid. Esterification was carried out as described for the ethyl ester of 3-methylphenylpropionic acid. B.p., 123° at 2 mm.; yield, 80 gm.; $n^{25}_{D} = 1.4870$; $D\frac{25}{4} = 0.969$.

$$[\alpha]^{25}_{D} = \frac{+3.34^\circ}{1 \times 0.969} = +3.45^\circ. \quad [M]^{25}_{D} = +7.60^\circ \text{ (homogeneous)}$$

3.955 mg. substance: 11.060 mg. CO_2 and 3.360 mg. H_2O.
$C_{14}H_{20}O_2$. Calculated. C 76.31, H 9.16
Found. " 76.25, " 9.50

Dextro-3-Phenyl-1-Hexanol—75 gm. of ethyl ester of 4-phenylcaproic acid (6), $[\alpha]^{25}_{D} = +3.45^\circ$, were dissolved in 300 cc. of absolute alcohol and this solution dropped into a suspension of 90 gm. of sodium in 450 cc. of boiling toluene with stirring. The carbinol was isolated as described for 3-phenylbutanol. B.p., 127° at 5 mm.; yield, 55 gm.; $n_D^{25} = 1.5101$; $D_4^{25} = 0.955$.

$$[\alpha]_D^{25} = \frac{+2.40^\circ}{1 \times 0.955} = +2.51^\circ. \quad [M]_D^{25} = +4.47^\circ \text{ (homogeneous)}$$

3.290 mg. substance: 9.760 mg. CO_2 and 3.030 mg. H_2O.
$C_{12}H_{18}O$. Calculated. C 80.83, H 10.18
Found. " 80.89, " 10.30

Dextro-1-Bromo-3-Phenylhexane—15 gm. of 3-phenyl-1-hexanol, $[\alpha]_D^{25} = +2.51^\circ$, were brominated by means of 25 gm. of phosphorus tribromide as described for 1-bromo-3-phenylbutane. B.p., 112° at 2 mm.; yield, 16 gm.; $n_D^{25} = 1.5250$; $D_4^{25} = 1.199$.

$$[\alpha]_D^{25} = \frac{+13.14^\circ}{1 \times 1.199} = +10.96^\circ. \quad [M]_D^{25} = +26.42^\circ \text{ (homogeneous)}$$

4.600 mg. substance: 10.080 mg. CO_2 and 2.935 mg. H_2O.
$C_{12}H_{17}Br$. Calculated. C 59.73, H 7.11
Found. " 59.74, " 7.14

Dextro-Ethyl-n-Propylphenylmethane—A Grignard reagent was prepared from 4 gm. of magnesium in dry ether and 30 gm. of 1-bromo-3-phenylhexane, $[\alpha]_D^{25} = +10.96^\circ$. The Grignard reagent was poured on ice and the hydrocarbon extracted and purified as described for methylethylphenylmethane. B.p., 105° at 27 mm.; yield, 19 gm.; $D_4^{24} = 0.863$.

$$[\alpha]_D^{24} = \frac{+0.58^\circ}{1 \times 0.863} = +0.67^\circ. \quad [M]_D^{24} = +1.09^\circ \text{ (homogeneous)}$$

3.605 mg. substance: 11.710 mg. CO_2 and 3.515 mg. H_2O.
$C_{12}H_{18}$. Calculated. C 88.81, H 11.19
Found. " 88.58, " 10.91

The preparation of this hydrocarbon was repeated starting with 24 gm. of 1-bromo-3-phenylhexane, $[\alpha]_D^{25} = +8.52^\circ$ (homogeneous). A hydrocarbon was obtained which had the following rotation.

$$[\alpha]_D^{25} = \frac{+0.48^\circ}{1 \times 0.861} = +0.56^\circ. \quad [M]_D^{25} = +0.91^\circ \text{ (homogeneous)}$$

4.331 mg. substance: 14.174 mg. CO_2 and 4.198 mg. H_2O.
$C_{12}H_{18}$. Calculated. C 88.81, H 11.19
Found. " 89.24, " 10.84

Dextro-2-Phenylbutyric Acid (*4*)—39 gm. of 2,2-methylphenylethylamine, $[\alpha]_D^{24} = \frac{+9.25°}{1 \times 0.938} = +9.86°$, $[M]_D^{24} = +13.31°$, were slowly dropped into an ether solution of nitrosyl chloride at $-50°$. The chloride was distilled under reduced pressure and then converted into a Grignard reagent. Carbon dioxide was passed into this reagent for about 15 minutes. The Grignard reagent was decomposed by pouring on ice and hydrochloric acid and extracting with ether. The ether solution was shaken with dilute potassium hydroxide, then separated and the alkaline solution extracted with ether. The solution was acidified, the organic acid extracted with ether, and distilled. B.p., 135° at 4 mm.; yield, 3.5 gm.

$$[\alpha]_D^{26} = \frac{+1.02°}{1 \times 1.066} = +0.96°. \quad [M]_D^{26} = +1.57° \text{ (homogeneous)}$$

3.875 mg. substance: 10.340 mg. CO_2 and 2.568 mg. H_2O.
$C_{10}H_{12}O_2$. Calculated. C 73.13, H 7.37
Found. " 72.76, " 7.41

Dextro-3-Phenylvaleric Acid (*5*)—57 gm. of 2,2-ethylphenylethylamine, $[\alpha]_D^{24} = -3.26°$, were chlorinated by means of nitrosyl chloride. Yield, 34 gm. A Grignard reagent was prepared from this chloride and magnesium in dry ether. The Grignard reagent was divided into two portions. The first portion was cooled in ice and dry carbon dioxide passed in for 15 minutes. The acid was isolated and purified as described for 2-phenylbutyric acid (4). B.p., 142° at 5 mm.; yield, 3 gm. (solid).

$$[\alpha]_D^{24} = \frac{+0.31° \times 100}{1 \times 0.542 \times 20} = +2.86°. \quad [M]_D^{24} = +5.10° \text{ (in benzene)}$$

5.671 mg. substance: 15.425 mg. CO_2 and 3.945 mg. H_2O.
$C_{11}H_{14}O_2$. Calculated. C 74.11, H 7.92
Found. " 74.17, " 7.79

Levo-Methylethylphenylmethane—The second portion of the Grignard reagent prepared above was poured on ice and hydrochloric acid. The hydrocarbon was isolated and purified as described for the first preparation of methylethylphenylmethane. B.p., 68° at 20 mm.; yield, 3 gm.

$$[\alpha]^{24}_{D} = \frac{-1.67^\circ}{1 \times 0.868} = -1.92^\circ. \quad [M]^{24}_{D} = -2.58^\circ \text{ (homogeneous)}$$

3.994 mg. substance: 13.080 mg. CO_2 and 3.770 mg. H_2O.
$C_{10}H_{14}$. Calculated. C 89.48, H 10.52
Found. " 89.30, " 10.56

Dextro-3-Phenylheptanoic Acid (7)—35 gm. of sodium were dissolved in 400 cc. of absolute alcohol and 240 gm. of ethyl malonate were added. To this were added 230 gm. of 1-bromo-3-phenylpentane, $[\alpha]^{25}_{D} = +11.05^\circ$, $[M]^{25}_{D} = +25.08^\circ$. The product was refluxed for 2 hours, and then the ester was isolated and hydrolyzed by boiling with 3 mols of potassium hydroxide dissolved in 80 per cent alcohol. The alcohol was evaporated and the sodium salt of the malonic acid was acidified with dilute sulfuric acid. The organic acid was extracted with ether, then the ether was evaporated. The acid was heated in a metal bath at 200° until carbon dioxide ceased coming off. It was then distilled. B.p., 162° at 1 mm.; yield, 152 gm.; $D\frac{25}{4} = 1.012$.

$$[\alpha]^{25}_{D} = \frac{+2.15^\circ}{1 \times 1.012} = +2.12^\circ. \quad [M]^{25}_{D} = +4.31^\circ \text{ (homogeneous)}$$

4.259 mg. substance: 11.720 mg. CO_2 and 3.085 mg. H_2O.
$C_{13}H_{18}O_2$. Calculated. C 75.67, H 8.80
Found. " 75.04, " 8.10

Dextro-Ethyl Ester of 3-Phenylheptanoic Acid (7)—152 gm. of 3-phenylheptanoic acid(7), $[\alpha]^{25}_{D} = +2.12^\circ$, $[M]^{25}_{D} = +4.37^\circ$, were dissolved in 400 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid were added. The product was heated 1 hour on a steam bath. The excess alcohol was distilled off and the residue washed with water, extracted with ether, and then distilled. B.p., 147° at 1 mm.; yield, 145 gm.; $D\frac{25}{4} = 0.969$.

$$[\alpha]^{25}_{D} = \frac{+1.02^\circ}{1 \times 0.969} = +1.05^\circ. \quad [M]^{25}_{D} = +2.46^\circ \text{ (homogeneous)}$$

3.735 mg. substance: 10.595 mg. CO_2 and 3.045 mg. H_2O.
$C_{15}H_{22}O_2$. Calculated. C 76.86, H 9.47
Found. " 77.35, " 9.12

Dextro-3-Phenylheptanol (7)—145 gm. of ethyl ester of 3-phenylheptanoic acid(7), $[\alpha]^{25}_{D} = +1.05^\circ$, $[M]^{25}_{D} = +2.46^\circ$, were dissolved in 500 cc. of absolute alcohol. This was slowly dropped into a suspension of 200 gm. of finely divided sodium in 1 liter of toluene, with vigorous stirring during addition. The excess sodium was destroyed by absolute alcohol, then water was added. The carbinol was extracted and then distilled. B.p., 145° at 1 mm.; yield, 110 gm.; $D\frac{25}{4} = 0.952$.

$$[\alpha]_D^{25} = \frac{+1.40^\circ}{1 \times 0.952} = +1.47^\circ. \quad [M]_D^{25} = +2.82^\circ \text{ (homogeneous)}$$

4.309 mg. substance: 12.860 mg. CO_2 and 3.945 mg. H_2O.
$C_{13}H_{20}O$. Calculated. C 81.18, H 10.49
Found. " 81.39, " 10.24

Dextro-3-Phenyl-7-Bromoheptane—100 gm. of 3-phenylheptanol(7), $[\alpha]_D^{25} = +1.47^\circ$, $[M]_D^{25} = +2.82^\circ$, were cooled in ice and 200 gm. of phosphorus tribromide were slowly added. The product was heated for 30 minutes on a steam bath, then poured into a flask containing ice. The halide was extracted with ether, then distilled. B.p., 131° at 1 mm.; yield, 103 gm.; $D\frac{25}{4} = 1.176$.

$$[\alpha]_D^{25} = \frac{+2.88^\circ}{1 \times 1.176} = +2.45^\circ. \quad [M]_D^{25} = +6.25^\circ \text{ (homogeneous)}$$

3.805 mg. substance: 8.490 mg. CO_2 and 2.440 mg. H_2O.
$C_{13}H_{19}Br$. Calculated. C 61.15, H 7.51
Found. " 60.84, " 7.17

Dextro-3-Phenylheptane—A Grignard reagent was prepared from 10 gm. of magnesium in 200 cc. of dry ether and 103 gm. of 3-phenyl-7-bromoheptane, $[\alpha]_D^{25} = +2.45^\circ$, $[M]_D^{25} = +6.25^\circ$. This was poured on ice and after dissolving the magnesium hydroxide with dilute hydrochloric acid, the hydrocarbon was extracted with ether. It was purified as previously described, then distilled. B.p., 105° at 16 mm.; yield, 24 gm.; $D\frac{25}{4} = 0.856$.

$$[\alpha]_D^{25} = \frac{+0.83^\circ}{1 \times 0.856} = +0.97^\circ. \quad [M]_D^{25} = +1.71^\circ \text{ (homogeneous)}$$

2.808 mg. substance: 9.108 mg. CO_2 and 2.915 mg. H_2O.
$C_{13}H_{20}$. Calculated. C 88.55, H 11.44
Found. " 88.45, " 11.61

Levo-4-Phenyloctanoic Acid (8)—13 gm. of sodium were dissolved in 150 cc. of absolute alcohol and 80 gm. of ethyl malonate were added. To this were added 125 gm. of 1-bromo-3-phenylhexane, $[\alpha]_D^{25} = -11.85°$. The product was refluxed 2 hours, then the ester was hydrolyzed by boiling with 150 gm. of potassium hydroxide dissolved in 1 liter of 80 per cent alcohol. The alcohol was evaporated, and the solution acidified with dilute sulfuric acid. The malonic acid was extracted with ether, and after evaporation of the ether was heated in a metal bath at 190° until carbon dioxide ceased coming off. It was then distilled. B.p., 170° at 1 mm.; yield, 85 gm.; $D_4^{25} = 0.999$.

$$[\alpha]_D^{25} = \frac{-0.82°}{1 \times 0.999} = -0.82°. \quad [M]_D^{25} = -1.80° \text{ (homogeneous)}$$

4.700 mg. substance: 13.185 mg. CO_2 and 3.865 mg. H_2O.

$C_{14}H_{20}O_2$.	Calculated.	C 76.31, H 9.16
	Found.	" 76.50, " 9.20

Levo-Ethyl Ester of 4-Phenyloctanoic Acid (8)—85 gm. of 4-phenyloctanoic acid (8), $[\alpha]_D^{25} = -0.82°$, were dissolved in 200 cc. of absolute alcohol and 8 cc. of concentrated sulfuric acid were added. The product was refluxed ½ hour, then the excess alcohol distilled, and the ester extracted with ether. It was then distilled. B.p., 152° at 1 mm.; yield, 93 gm.; $D_4^{25} = 0.958$.

$$[\alpha]_D^{25} = \frac{-0.47°}{1 \times 0.958} = -0.49°. \quad [M]_D^{25} = -1.22° \text{ (homogeneous)}$$

3.069 mg. substance: 8.633 mg. CO_2 and 2.720 mg. H_2O.

$C_{16}H_{24}O_2$.	Calculated.	C 77.36, H 9.75
	Found.	" 76.70, " 9.91

Levo-4-Phenyloctanol (8)—63 gm. of the ethyl ester of 4-phenyloctanoic acid(8), $[\alpha]_D^{25} = -0.48°$, were dissolved in 300 cc. of absolute alcohol and reduced by dropping into a suspension of 135 gm. of sodium in 675 cc. of toluene with vigorous stirring. Water was added after the sodium was dissolved. The carbinol was extracted with ether, then distilled. B.p., 147° at 1 mm.; yield, 47 gm.; $D_4^{25} = 0.941$.

$$[\alpha]_D^{25} = \frac{-1.04°}{1 \times 0.941} = -1.10°. \quad [M]_D^{25} = -2.28° \text{ (homogeneous)}$$

3.876 mg. substance: 11.575 mg. CO_2 and 3.730 mg. H_2O.

$C_{14}H_{22}O$.	Calculated.	C 81.48, H 10.76
	Found.	" 81.43, " 10.76

Levo-4-Phenyl-8-Bromooctane—47 gm. of 4-phenyloctanol(8), $[\alpha]_D^{25} = -1.10°$, were cooled in ice and 100 gm. of phosphorus tribromide were added. The product was heated for 15 minutes on a steam bath. It was then poured on ice and the halide extracted with ether and distilled. B.p., 146° at 1 mm.; yield, 43 gm.; $D_4^{25} = 1.109$.

$$[\alpha]_D^{25} = \frac{-2.42°}{1 \times 1.109} = -2.18°. \quad [M]_D^{25} = -5.87° \text{ (homogeneous)}$$

3.915 mg. substance: 9.020 mg. CO_2 and 2.805 mg. H_2O.

$C_{14}H_{21}Br$.	Calculated.	C 62.43, H 7.87
	Found.	" 62.82, " 8.01

Dextro-4-Phenyloctane—A Grignard reagent was prepared from 42 gm. of 4-phenyl-8-bromooctane, $[\alpha]_D^{25} = -2.18°$, and 5 gm. of magnesium in 100 cc. of ether. This was poured into a flask containing ice and dilute hydrochloric acid. The hydrocarbon was extracted with ether, dried, and distilled. It was purified as previously described, then distilled from sodium. B.p., 119° at 18 mm.; yield, 23 gm.; $D_4^{25} = 0.855$.

$$[\alpha]_D^{25} = \frac{+1.06°}{2 \times 0.855} = +0.62°. \quad [M]_D^{25} = +1.18° \text{ (homogeneous)}$$

2.781 mg. substance: 8.995 mg. CO_2 and 2.920 mg. H_2O.

$C_{14}H_{22}$.	Calculated.	C 88.34, H 11.66
	Found.	" 88.20, " 11.74

Dextro-2-Phenylcaproic Acid (*6*)—35 gm. of sodium were dissolved in 400 cc. of absolute alcohol and 240 gm. of ethyl malonate were added. To this were added 213 gm. of 1-bromo-3-phenylbutane, $[\alpha]_D^{25} = +6.03°$, $[M]_D^{25} = +12.84°$. The product was refluxed 2 hours on a water bath. The ester was then isolated and hydrolyzed by boiling with 3 mols of potassium hydroxide in 80 per cent alcohol. The excess alcohol was evaporated and the potassium salt of the malonic acid was acidified with 25 per cent sulfuric acid. This was extracted with ether and after evaporation of the ether was heated at 190° in a metal bath until CO_2 ceased coming off. The acid was then distilled. B.p., 156° at 1 mm.; yield, 138 gm.; $D_4^{25} = 1.022$.

$$[\alpha]_D^{25} = \frac{+2.05°}{1 \times 1.022} = +2.01°. \quad [M]_D^{25} = +3.86° \text{ (homogeneous)}$$

5.041 mg. substance: 13.696 mg. CO_2 and 3.718 mg. H_2O.
$C_{12}H_{16}O_2$. Calculated. C 74.95, H 8.39
Found. " 74.09, " 8.25

Dextro-Ethyl Ester of 2-Phenylcaproic Acid (*6*)—135 gm. of 2-phenylcaproic acid (6), $[\alpha]_D^{25} = +2.01°$, $[M]_D^{25} = +3.86°$, were dissolved in 300 cc. of absolute alcohol and 8 cc. of concentrated sulfuric acid were added. The product was refluxed on a steam bath for 30 minutes, the excess alcohol distilled off, and the ester extracted with ether. The ether was washed with dilute sodium carbonate solution, then the product distilled. B.p., 145° at 1 mm.; yield, 132 gm.; $D_4^{25} = 0.974$.

$$[\alpha]_D^{25} - \frac{+1.36°}{1 \times 0.974} \quad +1.40°. \quad [M]_D^{25} = +3.08° \text{ (homogeneous)}$$

2.795 mg. substance: 7.814 mg. CO_2 and 2.390 mg. H_2O.
$C_{14}H_{20}O_2$. Calculated. C 76.31, H 9.16
Found. " 76.23, " 9.56

Dextro-2-Phenylhexanol (*6*)—122 gm. of ethyl ester of 2-phenylcaproic acid(6), $[\alpha]_D^{25} = +2.01°$, $[M]_D^{25} = +3.86°$, were dissolved in 400 cc. of aboslute alcohol and reduced (in 40 gm. quantities) by slowly dropping into a suspension of 180 gm. of sodium in 900 cc. of boiling toluene with vigorous stirring. Water was added and the carbinol extracted with ether. It was then distilled. B.p., 127° at 1 mm.; yield, 75 gm.; $D_4^{25} = 0.967$.

$$[\alpha]_D^{25} = \frac{+1.90°}{1 \times 0.967} = +1.96°. \quad [M]_D^{25} = +3.46° \text{ (homogeneous)}$$

3.216 mg. substance: 9.625 mg. CO_2 and 2.880 mg. H_2O.
$C_{12}H_{18}O$. Calculated. C 80.83, H 10.18
Found. " 81.60, " 10.02

Dextro-2-Phenyl-1-Bromohexane—70 gm. of 2-phenylhexanol (6), $[\alpha]_D^{25} = +1.97°$, $[M]_D^{25} = +3.51°$, were cooled in ice and 100 gm. of phosphorus tribromide were added. The product was heated 15 minutes on a steam bath, poured on ice, and the halide extracted with ether. It was then distilled. B.p., 133° at 1 mm.; yield, 74 gm.; $D_4^{25} = 1.201$.

$$[\alpha]_D^{25} = \frac{+2.44°}{1 \times 1.201} = +2.03°. \quad [M]_D^{25} = +4.90° \text{ (homogeneous)}$$

5.066 mg. substance: 11.095 mg. CO_2 and 3.360 mg. H_2O.
$C_{12}H_{17}Br$. Calculated. C 59.73, H 7.11
Found. " 59.72, " 7.42

Dextro-2-Phenylhexane—A Grignard reagent was prepared from 40 gm. of 2-phenyl-1-bromohexane, $[\alpha]_D^{25} = +2.03°$, $[M]_D^{25} = +4.89°$, and 4 gm. of magnesium in 100 cc. of dry ether. The Grignard solution was poured on ice and hydrochloric acid. The hydrocarbon was extracted with ether, dried, and distilled from sodium. B.p., 100° at 22 mm.; yield, 21 gm.; $D_4^{25} = 0.855$.

$$[\alpha]_D^{25} = \frac{+1.68°}{1 \times 0.855} = +1.96°. \quad [M]_D^{25} = +3.18° \text{ (homogeneous)}$$

2.597 mg. substance: 8.430 mg. CO_2 and 2.655 mg. H_2O.
$C_{12}H_{18}$. Calculated. C 88.81, H 11.19
Found. " 88.52, " 11.44

Levo-1-Amino-2-Phenylpentane—25 gm. of *n*-propylphenylpropionic acid, $[\alpha]_D^{25} = -2.32°$ (homogeneous), were heated ½ hour on a steam bath with 100 gm. of thionyl chloride. The excess thionyl chloride was distilled off under reduced pressure. The residue in the distilling flask was converted into the amide by slowly dropping into 100 cc. of cold aqueous ammonium hydroxide solution with stirring. The amide was filtered and recrystallized from 50 per cent alcohol. It was dried *in vacuo*. To the dry amide was added 1 mol of bromine. The bromo amide was poured into 200 cc. of 10 per cent potassium hydroxide and heated on a steam bath for 6 hours. It was extracted with ether, and the ether shaken with dilute hydrochloric acid. The acid solution was extracted several times with ether, then made alkaline with potassium hydroxide. The amine was extracted with ether, dried, and distilled. B.p., 90° at 3 mm.; yield, 3 gm.

$$\alpha = \frac{-\,0.65^\circ}{2} = -\,0.33^\circ \text{ (homogeneous)}$$

$$[\alpha]_D^{25} = \frac{-\,0.10^\circ \times 100}{1 \times 20 \times 0.895} = -\,0.5^\circ \text{ (in 75 per cent alcohol)}$$

$$[\alpha]_D^{25} = \frac{-\,0.20^\circ \times 100}{2 \times 20 \times 1.23} = -\,0.4^\circ \text{ (hydrochloride in 75 per cent alcohol)}$$

3.413 mg. substance: 10.010 mg. CO_2 and 2.965 mg. H_2O.
$C_{11}H_{17}N$. Calculated. C 80.9, H 10.5
Found. " 80.0, " 9.8

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THE PARTIAL DEHYDROGENATION OF URSOLIC ACID

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In our studies on the partial dehydrogenation of β-amyrine[1] and hederagenin[2] and their derivatives it has been found that these substances show a close analogy in their behavior on heating with sulfur. Partial dehydrogenation may be accomplished in each case with the formation of cyclic sulfur compounds. These sulfur compounds exhibited similar properties and on oxidation gave rise to analogous sulfur-free substances which warrant the conclusion that analogous if not identical structures are present in both β-amyrine and hederagenin which are the points attacked by the sulfur. In the case of α-amyrine,[1] however, apparently only 1 mol of hydrogen was removed with the formation of a sulfur-free dehydro-α-amyrine which was characterized by its high positive rotation ($[\alpha]_D = +358°$). The divergent behavior of α-amyrine from the former substances indicated a definite structural difference.

In continuing our studies on the partial dehydrogenation of polyterpenes by an extension to other members of the group, another substance, ursolic acid, has been found to belong in the same category as α-amyrine, as regards its behavior towards sulfur. The present work was practically completed when the recent article of Sando[3] appeared. The latter confirmed the previous conclusion of van der Haar[4] that the three substances, malol, prunol, and ursolic acid of different origin are identical. Contrary, however, to van der Haar's formulation of $C_{31}H_{50}O_3$, Sando has concluded from numerous analyses that $C_{30}H_{48}O_3$ appears to be the correct formula. In reporting our own analytical results we are giving this conclusion merely provisional acceptance.

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[1] Jacobs, W. A., and Fleck, E. E., *J. Biol. Chem.*, **88**, 137 (1930).

[2] Jacobs, W. A., and Fleck, E. E., *J. Biol. Chem.*, **88**, 153 (1930).

[3] Sando, C. E., *J. Biol. Chem.*, **90**, 477 (1931).

[4] van der Haar, A. W., *Rec. trav. chim. Pays-Bas*, **43**, 542, 548 (1924).

In our first experiments the attempt was made to employ ursolic acid and its methyl ester directly for partial dehydrogenation with sulfur. Extensive carbonization, however, was found to occur and following our experience in other cases we turned to derivatives of the ester in which the apparently disturbing hydroxyl was removed. In one case *ursolic methyl ester benzoate* was employed. On being heated with sulfur a substance was formed apparently with the loss of 1 mol of hydrogen. The resulting *dehydroursolic methyl ester benzoate* was characterized by a high positive rotation ($[\alpha]_D = +241°$).

In another series of experiments the hydroxyl group of ursolic acid was shown to be of secondary character since the ester on oxidation in acetic acid solution with chromic acid yielded a ketone, *ursonic methyl ester*, which was characterized by its *oxime*. If the oxidation mixture contained sulfuric acid (Kiliani CrO_3 solution), the oxidation proceeded further with the formation apparently of a *diketone*, $C_{30}H_{46}O_4$. From the latter, however, only a *monooxime* could be obtained. The keto group of ursonic methyl ester was readily reduced by the Clemmensen method to CH_2 with the formation of *ursanic methyl ester* in which the ester group was still very resistant to saponification. Only by vigorous treatment with alkali could it be saponified to *ursanic acid.*

When ursanic methyl ester was heated with sulfur at 230–240° partial dehydrogenation occurred and from the reaction mixture a substance was obtained which analysis indicated to possess the formula $C_{30}H_{46}O_2$ and which had been formed apparently by the loss of 1 mol of hydrogen. This substance, *dehydroursanic methyl ester*, like dehydroursolic methyl ester benzoate and α-amyrine, possessed a strong positive rotation, $[\alpha]_D = +288°$. Like its parent ester, it was saponified with difficulty to *dehydroursanic acid.*

It appears, therefore, that triterpene derivatives, as regards their behavior on partial dehydrogenation with sulfur, may be separated at least into two categories. The difference in structure responsible for this divergent behavior of the two groups is now under investigation.

EXPERIMENTAL

Ursolic Acid—Crude ursolic acid which melted at 279–280° (corrected) was isolated in the form of colorless needles from *Arctostaphylos uva-ursi* leaves essentially by the method of Dodge.[5]

For molecular weight determinations and analysis the ursolic acid was purified, through the sodium salt, by the method of Sando.[3] The final product melted at 284–285° (corrected).[6]

4.040 mg. substance: 3.875 mg. H_2O, 11.658 mg. CO_2.
3.925 " " : 3.800 " " 11.350 " "
$C_{30}H_{48}O_3$. Calculated. C 78.88, H 10.61
Found. (a) " 78.70, " 10.73
(b) " 78.86, " 10.83

Ursolic Methyl Ester—When crude ursolic acid was esterified in ether solution with diazomethane complete solution occurred at once. After removal of the solvent the residue was fractionated from acetone. Two substances which differed in solubility were separated by this procedure. The less soluble substance formed needles which melted at 221–222°.

$[\alpha]_D^{27} = +59°$ (c = 1.027 in pyridine).
5.075 mg. substance: 4.965 mg. H_2O, 14.947 mg. CO_2.
5.225 " " : 5.130 " " 15.390 " "
5.565 " " : 1.395 " AgI.
Found. (a) C 80.31, H 10.95
(b) " 80.33, " 10.98
(c) OCH_3 3.31

Molecular weight determination was made according to the method of Rast. 28.130 mg. of camphor: 2.663 mg. of anhydrous substance, $\Delta = 9.4°$. Molecular weight found, 453.

This substance was recovered unchanged after boiling with 10 per cent alcoholic KOH for 4 hours. The above methoxyl figure is about one-half of that for ursolic methyl ester itself. This observation, together with the fact that the substance yields on oxidation with CrO_3 about one-half of the amount of ursonic methyl ester given by pure ursolic methyl ester, indicates that the substance is possibly a molecular association compound of ursolic methyl ester and some other substance of the same molecular order. The substance has not been investigated further.

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[5] Dodge, F., *J. Am. Chem. Soc.*, **40**, 1932 (1918).

[6] When the uncorrected melting point of this substance was taken in the usual manner it was found to be 275–276°. The above value was found after correcting for projecting thermometer stem.

The more soluble substance yielded a substance which melted at 110–120° and corresponded in all respects with the monohydrate of ursolic methyl ester as previously described.[3,7] The yield was 5.5 gm. from 10 gm. of crude ursolic acid.

$[\alpha]_D^{24}$ = +58° (c = 1.030 in pyridine).
4.345 mg. substance: 4.190 mg. H_2O, 12.615 mg. CO_2.
4.270 " " : 4.025 " " 12.380 " "
5.250 " " : 2.500 " AgI.
$C_{31}H_{50}O_3$. Calculated. C 79.08, H 10.71, OCH_3 6.59
Found. (a) " 79.19, " 10.79
(b) " 79.07, " 10.55
(c) OCH_3 6.30

Benzoate of Ursolic Methyl Ester—Ursolic methyl ester was benzoylated in pyridine solution with benzoyl chloride. The benzoate formed needles from acetone which melted at 212–213°. After the melt was allowed to resolidify it melted at 235–236°.

By the Liebermann-Burchard procedure it gave a pink color which changed to a greenish brown when the solution was warmed.

$[\alpha]_D^{24}$ = +70° (c = 1.035 in pyridine).
4.310 mg. substance: 3.630 mg. H_2O, 12.520 mg. CO_2.
4.730 " " : 3.995 " " 13.790 " "
6.072 " " : 2.435 " AgI.
$C_{38}H_{54}O_4$. Calculated. C 79.38, H 9.47, OCH_3 5.39
Found. (a) " 79.22, " 9.42
(b) " 79.52, " 9.46
(c) OCH_3 5.30

Dehydroursolic Methyl Ester Benzoate—A mixture of 0.5 gm. of ursolic methyl ester benzoate and an equal amount of sulfur was heated at 230–240° for 3½ hours in an atmosphere of nitrogen. The reaction mass was extracted with ether and the extract was evaporated to dryness. The residue was dissolved in a small amount of ether and the sulfur which remained was filtered off. After concentrating the filtrate this process was repeated until complete solution of the residue occurred. The final residue was

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[7] Power, F. B., and Moore, C. W., *J. Chem. Soc.*, **97**, 1106 (1910). van der Haar, A. W., *Rec. trav. chim. Pays-Bas*, **43**, 375 (1924).

then dissolved in alcohol and the solution was decolorized with Darco. For final purification it was recrystallized from acetone from which it crystallized as needles which melted at 210–212°.

The cholesterol test showed a deep purple color which changed to a dark brown when the solution was warmed.

$[\alpha]_D^{24} = +241°$ (c = 1.010 in pyridine).
4.963 mg. substance: 3.990 mg. H_2O, 14.525 mg. CO_2.
4.968 " " : 4.102 " " 14.527 " "
$C_{38}H_{52}O_4$. Calculated. C 79.66, H 9.15
Found. (a) " 79.82, " 9.01
(b) " 79.75, " 9.24

Ursonic Methyl Ester—A solution of 5 gm. of ursolic methyl ester in 50 cc. of acetic acid was added to an acetic acid solution of 1 gm. of CrO_3, and the mixture was heated on the steam bath for ½ hour. The product was precipitated with 3 volumes of water. For purification the precipitate was recrystallized from acetone from which it separated in the form of prisms which melted at 192–193°. Ursonic methyl ester is more soluble in ether and ligroin (80–90°) than in petrolic ether, alcohol, and acetone.

The cholesterol test gave a pink color which on warming changed to a light blue becoming finally a light green.

$[\alpha]_D^{23} = +84°$ (c = 1.010 in pyridine).
4.640 mg. substance: 4.285 mg. H_2O, 13.535 mg. CO_2.
4.352 " " : 4.002 " " 12.700 " "
5.175 " " : 2.685 " AgI.
$C_{31}H_{48}O_3$. Calculated. C 79.42, H 10.33, OCH_3 6.61
Found. (a) " 79.56, " 10.33
(b) " 79.59, " 10.29
(c) OCH_3 6.85

Ursonic Methyl Ester Oxime—The oxime was prepared in the usual manner in alcoholic solution. It formed needles from methyl alcohol which melted at 243–244°.

3.850 mg. substance: 3.620 mg. H_2O, 10.920 mg. CO_2.
4.080 " " : 2.030 " AgI.
6.017 " " : 0.166 cc. N_2 (30° and 752.7 mm.).
$C_{31}H_{49}O_3N$. Calculated. C 76.96, H 10.19, OCH_3 6.41, N 2.90
Found. (a) " 77.35, " 10.52
(b) OCH_3 6.56
(c) N 3.08

The Diketone, $C_{31}H_{46}O_4$—A solution of 0.5 gm. of ursolic methyl ester in 0.5 cc. of chloroform and 100 cc. of acetic acid was treated with 25 cc. (a large excess) of Kiliani's chromic acid solution. The temperature of the reaction was not allowed to rise above 28°. At the end of ½ hour the solution was diluted with 3 volumes of water and then extracted with ether. The extract was washed with dilute alkali. The alkaline extract when acidified gave a small amount of non-crystallizable gum.

The ether solution of the neutral portion gave a residue which was crystallized from dilute alcohol. It separated as stout prisms which melted at 176–177°.

The cholesterol test gave an orange-yellow color which deepened on warming.

$[\alpha]_D^{28} = +128°$ (c = 1.017 in pyridine).
3.486 mg. substance: 2.995 mg. H_2O, 9.855 mg. CO_2.
3.615 " " : 3.040 " " 10.210 " "
6.225 " " : 3.060 " AgI.
$C_{31}H_{46}O_4$. Calculated. C 77.12, H 9.60, OCH_3 6.42
Found. (a) " 77.10, " 9.61
(b) " 77.01, " 9.41
(c) OCH_3 6.48

Monooxime of Diketone, $C_{31}H_{46}O_4$—This was prepared in the usual manner in alcoholic solution. The oxime was recrystallized from methyl alcohol from which it separated as flat prisms which melted at 210–211°.

3.877 mg. substance: 3.380 mg. H_2O, 10.680 mg. CO_2.
4.365 " " : 2.100 " AgI.
6.405 " " : 0.169 cc. N_2 (29° and 753.5 mm.).
$C_{31}H_{47}O_4N$. Calculated. C 74.80, H 9.53, OCH_3 6.23, N 2.81
Found. (a) " 75.13, " 9.75
(b) OCH_3 6.40
(c) N 2.96

Ursanic Methyl Ester—A solution of 2 gm. of ursonic methyl ester in 320 cc. of acetic acid and 80 cc. of HCl (1.19) was added to 50 gm. of amalgamated zinc. The mixture was refluxed gently for 1 hour. The reaction mass was diluted with 3 volumes of water and the resulting precipitate was collected with water. For analysis this product was recrystallized from methyl alcohol. It separated as needles which melted at 117–118°. Ursanic methyl ester is very soluble in ether, acetone, and ligroin. It is less soluble in alcohol and least soluble in methyl alcohol.

The cholesterol test gave a pink color which changed to light brown on warming.

$[\alpha]_D^{29}$ = +65° (c = 1.020 in pyridine).
4.218 mg. substance: 4.260 mg. H_2O, 12.683 mg. CO_2.
3.777 " " : 3.800 " " 11.320 " "
4.393 " " : 2.145 " AgI.
$C_{31}H_{50}O_2$. Calculated. C 81.87, H 11.09, OCH_3 6.82
Found. (a) " 82.01, " 11.30
(b) " 81.74, " 11.25
(c) OCH_3 6.45

Ursanic Acid—A solution of 0.1 gm. of ursanic methyl ester in 10 cc. of 20 per cent alcoholic KOH was heated in a sealed tube at 120–130° for 3 hours. The precipitate of salt and unchanged ester, which separated when the reaction mixture was diluted with 3 volumes of water, was filtered and dried. This dried material was extracted several times with dry ether in order to remove unchanged ester. The insoluble potassium salt was suspended in ether and brought into solution by the addition of a few drops of HCl. The washed ether solution was evaporated and the residue was recrystallized from dilute acetone. Ursanic acid separated as needles which melted at 223–225°.

3.967 mg. substance: 3.990 mg. H_2O, 11.853 mg. CO_2.
3.485 " " : 3.378 " " 10.395 " "
$C_{30}H_{48}O_2$. Calculated. C 81.74, H 10.99
Found. (a) " 81.49, " 11.25
(b) " 81.35, " 10.85

Dehydroursanic Methyl Ester—A mixture of 0.5 gm. of ursanic methyl ester and an equal amount of sulfur was heated in an atmosphere of nitrogen at a temperature of 230–240° for 2½ hours. At the end of this period H_2S production had practically ceased. The reaction mass was extracted with ether and the residue obtained after removal of the ether was distilled at 2 mm. The distillation was interrupted when the temperature of the metal bath had reached 275°. At this point practically all of the sulfur had distilled. When the temperature of the bath was maintained between 275–310° a resin distilled. This resin crystallized when dissolved in methyl alcohol. Repeated recrystallizations from methyl alcohol yielded prisms which melted at 110–111°. The yield was about 20 per cent.

The cholesterol test gave a deep violet color which changed to a dark brown when the solution was warmed.

$[\alpha]_D^{24} = +288°$ (c = 1.020 in pyridine).
4.623 mg. substance: 4.420 mg. H_2O, 13.932 mg. CO_2.
3.943 " " : 3.780 " " 11.890 " "
4.985 " " : 2.450 " AgI.
$C_{31}H_{48}O_2$. Calculated. C 82.23, H 10.70, OCH_3 6.85
Found. (a) " 82.19, " 10.70
(b) " 82.24, " 10.73
(c) OCH_3 6.50

Dehydroursanic Acid—A solution of 0.1 gm. of dehydroursanic methyl ester in 10 cc. of 20 per cent alcoholic KOH was heated in a sealed tube at 120–130° for 3 hours. The reaction mixture was treated in the same manner as given in the case of ursanic acid. Final recrystallization from dilute acetone yielded needles which melted at 174–176°.

3.770 mg. substance: 3.560 mg. H_2O, 11.355 mg. CO_2.
$C_{30}H_{46}O_2$. Calculated. C 82.13, H 10.57
Found. " 82.15, " 10.57

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STROPHANTHIN

XXIV. ISOMERIC HEXAHYDRODIANHYDROSTROPHANTHIDINS AND THEIR DERIVATIVES

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The correlation of the digitalis aglucones with periplogenin and strophanthidin has recently been described.[1] This was accomplished by the use of certain derivatives of the iso compounds of these substances. For some time before this group of derivatives was used another method of approach had been attempted, based upon transformations with dianhydrostrophanthidin. Starting with this unsaturated substance the plan had been to hydrogenate its three double bonds with the production of a saturated hexahydrodianhydrostrophanthidin. The next step was the reduction of the carbonyl group still present in the substance to methyl. At this point it was hoped that comparisons with similar substances obtained from other cardiac aglucones might be feasible. A part of our experience with this program has already been presented.[2] It was found that the catalytic hydrogenation with palladium black of dianhydrostrophanthidin under the conditions which we had used proceeded beyond the stage desired and that an oxygen atom was removed with the formation of a mixture of isomeric octahydrotrianhydrostrophanthidins. This result was correctly interpreted to be due to the simultaneous reduction of the lactol form of the hydroxyaldehyde to the saturated alkylene oxide.

Windaus, Reverey, and Schwieger,[3] who were simultaneously occupied with the same problem, later published a report of their own studies. Among other things it was found that dianhydrostrophanthidin, depending upon the catalyst used (platinum black containing hydrogen or oxygen), could be reduced to a hexahydrodianhydrostrophanthidin, $C_{23}H_{34}O_4$, or an octahydro derivative, $C_{23}H_{36}O_4$. In experiments which we have since attempted with various platinum catalysts we have always noted a steady absorption, of course with definite breaks in the curve, of 4 mols of hydrogen with the formation of mixtures of isomeric octahydrotrianhydrostrophanthidins, thus repeating our earlier experience with palladium black. We have not, therefore, been able to duplicate the conditions which were employed by Windaus and coworkers. For this we have no explanation.

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[1] Jacobs, W. A., and Elderfield, R. C., *J. Biol. Chem.*, **91**, 625 (1931); **92**, 313 (1931).

[2] Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **63**, 123 (1925).

[3] Windaus, A., Reverey, G., and Schwieger, A., *Ber. chem. Ges.*, **58**, 1509 (1925).

In order to prevent the reduction of the lactol to the oxide we turned to the direct hydrogenation of the ethylal of oxidodianhydrostrophanthidin in a neutral solvent in which the ethylal group is stable. In alcoholic solution this substance readily absorbed 3 mols of hydrogen with the formation of a mixture of more soluble isomeric saturated substances. Among these a substance preponderated of melting point 128° and of $[\alpha]_D = +21°$, which has been designated the *ethylal of α-hexahydrooxidodianhydrostrophanthidin.*[4] On hydrolysis this substance readily yielded the hydroxyaldehyde, *α-hexahydrodianhydrostrophanthidin.* The latter was found to react both in the free aldehydic and in the lactol form. From the fact that in the preparation of its *oxime* more vigorous treatment was necessary than has usually been required by this class of substances the impression was obtained that the lactol form is the more stable form. When the attempt was made to reduce the substance by the Clemmensen method the desired desoxo derivative was not produced. Instead, the substance was reduced in the lactol form with the production of a mixture of isomeric *octahydrotrianhydrostrophanthidins* which resembled in properties the mixture obtained by catalytic hydrogenation. On the other hand, on benzoylation the secondary alcoholic group of the aldehydic form of dianhydrostrophanthidin was acylated since the resulting *dianhydrostrophanthidin benzoate* formed an *oxime.*

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[4] The designation of α and β in these cases is arbitrary and bears no relationship to the designation of α and β previously given to the isomeric isostrophanthidins and their derivatives.

With the hope that the benzoyl group would prevent oxide formation the attempt was then made to reduce this benzoate by the Clemmensen method. Although a reaction occurred, the results obtained were inconclusive. The attempt was also made to prepare a semicarbazone for subsequent reduction by the Wolff method. The semicarbazone formation, however, proved to be very incomplete, apparently due to the persistence of the substance in the lactol form under the conditions used. Further investigation of this phase of the problem was, therefore, discontinued especially in view of the greater promise of the work in the isostrophanthidin series, which has since been published.

In the course of the investigation of α-hexahydrodianhydrostrophanthidin, its behavior on oxidation with chromic acid was studied. This reaction was found to proceed smoothly with the formation of a neutral *dilactone*, $C_{23}H_{32}O_4$, from the lactol form. This oxidation product consisted mainly of a substance which differed in melting point and properties from a previously described hexahydrodilactone[5] which was obtained by hydrogenation of the dianhydrodilactone, $C_{23}H_{26}O_4$. A minor portion of the above oxidation mixture, however, was separated because of its sparing solubility. It appeared to be identical with the earlier saturated dilactone. The formation, therefore, of such isomeric dilactones on oxidation suggested the possibility that the so called α-hexahydrodianhydrostrophanthidin is not entirely homogeneous. This point will, however, be again referred to.

Since the direct hydrogenation of the three double bonds in a substance like the ethylal of oxidodianhydrostrophanthidin is practically certain to give rise to a mixture of isomers another graduated procedure was studied in which each double bond was individually produced and hydrogenated. This was made possible by starting with dihydrostrophanthidin. The latter as already reported[6] readily loses 1 mol of water with the formation of anhydrodihydrostrophanthidin. This substance was hydrogenated with difficulty to form *dihydroanhydrodihydrostrophanthidin*. The remaining tertiary hydroxyl group was readily removed from this substance by alcoholic hydrogen chloride with the formation of the *ethylal of dihydrooxidodianhydrodihydrostrophanthi-*

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[5] Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **65**, 504 (1925).

[6] Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, **74**, 791 (1927).

din. The latter on hydrogenation absorbed 1 mol of hydrogen with the formation of the saturated *ethylal of β-hexahydrooxidodianhydrostrophanthidin.* This substance of melting point 131° and of rotation $[\alpha]_D = +13°$ resembles very closely in all other properties the previous α-isomer. On hydrolysis *β-hexahydrodianhydrostrophanthidin* was formed. Like its α-isomer this substance also reacted in both the free aldehydic and the lactol forms. It yielded an *oxime.* Its monobenzoate proved to be likewise a derivative of the aldehydic form since this derivative also gave an *oxime.* Attempts to reduce the benzoate by the Clemmensen method were again inconclusive. Like its α-isomer, β-hexahydrodianhydrostrophanthidin was oxidized by chromic acid in the lactol form to give a hexahydrodilactone which again proved to be a mixture. In this case the high melting, sparingly soluble substance predominated which was encountered as a minor fraction of the oxidation product from the α-isomer. This observation brings again in question the homogeneity of the above β-hydrogenation product. It is conceivable though that partial isomerization may have occurred under the conditions of the oxidation. Both of the isomeric hexahydrodianhydrostrophanthidins as well as the dilactones obtained from them were found to be transformed readily into mixtures of isomers on heating with dilute hydrochloric acid.

From our experience with the hydrogenation of the unsaturated strophanthidin derivatives and from that of Windaus and coworkers with the digitalis aglucones,[7] it is apparent that this method of approach for the correlation of the cardiac aglucones has proved to be too complicated to be practicable. As we have already shown, the iso derivatives of these aglucones have presented much more favorable opportunities. The further use of the iso compounds is being applied in the case of certain other aglucones of the strophanthidin group for purposes of correlation.

EXPERIMENTAL

Part I

Ethylal of α-Hexahydrooxidodianhydrostrophanthidin—A suspension of the ethylal of oxidodianhydrostrophanthidin in alcohol was shaken with purified hydrogen and 0.1 gm. of platinum oxide catalyst. The absorption, at first rapid, gradually decreased so that in some cases it was necessary after a day or so to add a further 0.1 gm. of catalyst to complete the reaction. In different experiments from 1 to 6 days were found necessary for the absorbtion of the required 3 mols of hydrogen. The resulting clear solution no longer gave a nitroprusside reaction. The filtrate from the catalyst showed no appreciable acidity so that no evidence was obtained of the formation of desoxy acid during the hydrogenation of the unsaturated lactone group. The filtrate on concentration yielded successive crops of crystals. On fractionation from alcohol about two-thirds of the material was obtained with constant melting point and rotation. It formed needles which were soluble in the usual solvents and melted at 128–129°.

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[7] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **86**, 200 (1930).

$[\alpha]_D^{20} = +21°$ (c = 1.0 in 95 per cent alcohol).
3.880 mg. substance: 3.290 mg. H_2O, 10.580 mg. CO_2.
$C_{25}H_{38}O_4$. Calculated. C 74.57, H 9.52
Found. " 74.37, " 9.49

The mother liquors yielded additional crystalline material, of somewhat lower melting point and lower rotation, which consisted of stereoisomers.

The above ethylal is isomeric with the substance obtained by the stepwise dehydration and hydrogenation of dihydrostrophanthidin. It appeared possible that this isomerism was due to the interposition of the action of HCl at one of the steps in the series of reactions giving the ethylal from dihydrostrophanthidin. To test this possibility the attempt was made to convert the above ethylal into this latter isomer by refluxing for 30 minutes with 5 per cent absolute alcoholic hydrogen chloride. The solution was then poured into $NaHCO_3$ solution and the precipitate was collected with water. The material proved to be a mixture of substances which crystallized slowly from the concentrated alcoholic solution. This substance proved to be very soluble and melted draggily at 110–115°. Although after recrystallization this melting point could be raised giving a substance of $[\alpha]_D = +14°$, this was accomplished only by a profitless use of material. Although the above ethylal is definitely isomerized by acid the reaction is not a homogeneous one leading to the isomer derived from dihydrostrophanthidin.

α-Hexahydrodianhydrostrophanthidin—0.5 gm. of the ethylal was dissolved by gentle warming in 4 cc. of 50 per cent acetic acid. After being chilled a precipitate formed on dilution. On recrystallization from methyl alcohol the hydroxyaldehyde formed fine needles which softened at about 112° to a resinous mass of bubbles that finally melted at 178–180°.

$[\alpha]_D^{25} = +41°$ (c = 1.055 in chloroform).

For analysis the substance was dried at 80° and 15 mm.

5.147 mg. substance: 4.295 mg. H_2O, 13.960 mg. CO_2.
$C_{23}H_{34}O_4$. Calculated. C 73.74, H 9.16
Found. " 73.97, " 9.34

This hydroxyaldehyde is apparently a mixture of the lactol and free aldehydic forms. The formation of an *oxime* did not proceed with the usual readiness. For a complete reaction it was found necessary to heat the hydroxyaldehyde in alcoholic solution with hydroxylamine hydrochloride and sodium acetate in a sealed tube at 100° for 6 hours. The reaction product obtained on dilution was recrystallized from alcohol and formed leaflets which melted at 258–260° after preliminary softening.

4.498 mg. substance: 3.585 mg. H_2O, 11.723 mg. CO_2.
6.520 " " : 0.211 cc. N (28°, 754.5 mm.).
$C_{23}H_{35}O_4N$. Calculated. C 70.90, H 9.07, N 3.59
Found. " 71.07, " 8.92
" " 3.65

Octahydrotrianhydrostrophanthidin—When the attempt was made to apply the Clemmensen method for the reduction of α-hexahydrodianhydrostrophanthidin to the desoxo compound the reaction took a different course. The substance was reduced directly in the lactol form to the saturated oxide. For this purpose the ethylal was directly used because of its prompt preliminary hydrolysis.

A solution of 0.1 gm. of the ethylal in 5 cc. of acetic acid was treated with 2.5 gm. of amalgamated zinc and 2.5 cc. of HCl (1.19). The mixture was refluxed for ½ hour. The reaction product crystallized on dilution. On fractionation from methyl alcohol it was found to be a mixture of isomers. The first crop which was still apparently a mixture melted at 196–202° and gave a rotation of $[\alpha]_D^{26} = +61°$ (c = 0.990 in chloroform). This material closely resembles the substance previously obtained by catalytic hydrogenation of dianhydrostrophanthidin.[2] No attempt was made to purify it exhaustively.

5.120 mg. substance: 4.470 mg. H_2O, 14.540 mg. CO_2.
$C_{23}H_{34}O_3$. Calculated. C 77.03, H 9.57
Found. " 77.45, " 9.77

The oxidic character of the substance was shown by the fact that it was not attacked under the usual conditions by permanganate or chromic acid. The above mother liquors yielded lower melting isomeric material with similar properties.

Benzoyl-α-Hexahydrodianhydrostrophanthidin—The benzoate was prepared by benzoylation in pyridine solution. It formed very sparingly soluble crystalline aggregates from alcohol which melted at 213–215°.

4.520 mg. substance: 3.250 mg. H_2O, 12.475 mg. CO_2.
$C_{30}H_{38}O_5$. Calculated. C 75.27, H 8.01
Found. " 75.27, " 8.05

This substance was shown to be the benzoyl derivative of the hydroxyaldehyde and not its lactol form by its ready conversion into an *oxime*. This derivative was made in the usual manner in alcoholic solution. It formed needles from alcohol which melted at 242–243°.

4.513 mg. substance: 3.252 mg. H_2O, 12.043 mg. CO_2.
6.548 " " : 0.174 cc. N (24°, 760 mm.).
$C_{30}H_{39}O_5N$. Calculated. C 72.97, H 7.97, N 2.84
Found. " 72.77, " 8.06
" " 3.13

In all attempts to prepare a semicarbazone from the benzoate, difficulty was experienced in bringing the reaction to completion. The substance recovered was never homogeneous and no satisfactory means of purification were found.

Hexahydrodilactone, $C_{23}H_{32}O_4$—Since hydrolysis of the ethylal group readily occurred, the ethylal was directly employed for oxidation to the dilactone. 0.5 gm. of the α-hexahydroethylal was dissolved in 10 cc. of acetic acid and the solution was treated with 2.4 cc. of Kiliani CrO_3 solution. A prompt reaction occurred. After 10 minutes the mixture was diluted. Fractionation from alcohol was necessary in order to remove small amounts of higher melting, less soluble, isomeric material. The majority was recovered as narrow prisms or needles from alcohol, which melted at 192–194°. The homogeneity of this fraction is perhaps doubtful.

$[\alpha]_D^{26} = +19.4°$ (c = 0.515 in chloroform).
4.305 mg. substance: 3.440 mg. H_2O, 11.685 mg. CO_2.
$C_{23}H_{32}O_4$. Calculated. C 74.14, H 8.67
Found. " 74.02, " 8.94

The small fraction of less soluble isomer obtained during the above fractionation was recrystallized from acetone. It formed needles and short stout prisms which melted at 265–267°.

$[\alpha]_D^{28} = +13°$ (c = 1.10 in chloroform).
4.562 mg. substance: 3.580 mg. H_2O, 12.420 mg. CO_2.
Found. C 74.25, H 8.78

This substance is apparently identical with the previously reported hexahydrodilactone[6] obtained by hydrogenation of the dianhydrodilactone, $C_{23}H_{26}O_4$. Since H_2SO_4 was present in the Kiliani CrO_3 solution used above, it appeared possible that the small amount of high melting isomer might have been due to the isomerizing effect of the strong acid. This appears to have been confirmed by the following experiment.

0.2 gm. of the hexahydrodilactone melting at 192–194° was refluxed in a mixture of 25 cc. of acetic acid and 5 cc. of HCl (1.19) for 45 minutes. Crystalline material was obtained on dilution. After several recrystallizations from methyl alcohol and finally from acetone it melted at 261–264°.

$[\alpha]_D^{26} = +13.2°$ (c = 0.740 in chloroform).
4.597 mg. substance: 3.565 mg. H_2O, 12.510 mg. CO_2.
Found. C 74.17, H 8.67

On this occasion we have checked more fully the previously reported[6] direct hydrogenation of the dianhydrodilactone, $C_{23}H_{26}O_4$. The reaction product has been found to be also a mixture of isomers. As previously given repeated recrystallization from alcohol gave the substance melting at 265–267° with $[\alpha]_D = +14°$. The mother liquors from this material yielded more soluble fractions from which an isomer has now been separated by taking advantage of its greater solubility in ether. It was finally obtained by concentration of the ether solution as characteristic six-sided platelets which melted at 204°. This melting point depended upon the solvent since when alcohol was used it melted at 196–199°.

$[\alpha]_D^{26} = +18°$ (c = 1.03 in chloroform).
5.320 mg. substance: 4.185 mg. H_2O, 14.495 mg. CO_2.
Found. C 73.95, H 8.80

Although this hexahydrodilactone resembles so closely in properties the above low melting oxidation product of the hexahydroethylal it must be a different substance. Contrary to the latter, it was not converted into a high melting isomer on boiling in acetic acid containing HCl.

Part II

Benzoyl Monoanhydrodihydrostrophanthidin—This substance was prepared by benzoylation of monoanhydrodihydrostrophanthidin[6] in pyridine solution. It formed stout prisms which melted at 190–192°.

0.0963 gm. substance: 0.0644 gm. H_2O, 0.2570 gm. CO_2.
$C_{30}H_{36}O_6$. Calculated. C 73.13, H 7.37
Found. " 72.87, " 7.48

Dihydromonoanhydrodihydrostrophanthidin—The double bond of monoanhydrodihydrostrophanthidin was found to be unusually difficult to hydrogenate. Only when very carefully performed was the operation a smooth and complete affair. The best results were obtained by the use of material which had been repeatedly recrystallized from alcohol that had been distilled repeatedly in a glass jointed apparatus.

A suspension of 6.2 gm. of substance in 400 cc. of alcohol was shaken with hydrogen and 0.2 gm. of the platinum catalyst of Adams and Shriner. In this instance absorption corresponding to 1 mol required several hours for completion. On another occasion 1½ hours sufficed while in other attempts it was necessary to add more catalyst and even then several days were required before the absorption of 1 mol was completed.

The saturated substance formed leaflets from methyl alcohol which melted at 217–219°.

$[\alpha]_D^{20} = +28°$ (c = 1.000 in pyridine).
0.1005 gm. substance: 0.0789 gm. H_2O, 0.2613 gm. CO_2.
$C_{23}H_{34}O_5$. Calculated. C 70.72, H 8.78
Found. " 70.89, " 8.79

Ethylal of Dihydrooxidodianhydrodihydrostrophanthidin—The preceding hydroxyaldehyde was refluxed for 30 minutes in 10 parts of absolute alcohol containing 5 per cent of hydrogen chloride. On being cooled the mixture formed a thick mass of crystals. The ethylal formed stout needles from alcohol and melted at 174–176°.

$[\alpha]_D^{25} = -108°$ (c = 0.950 in chloroform).
2.946 mg. substance: 2.346 mg. H_2O, 8.075 mg. CO_2.
$C_{25}H_{36}O_4$. Calculated. C 74.94, H 9.06
Found. " 74.74, " 8.91

Dihydrodianhydrodihydrostrophanthidin—On diluting an alcoholic solution of the above ethylal with water containing dilute hydrochloric acid hydrolysis occurred and the free hydroxyaldehyde slowly separated as arborescent masses of prisms which were characterized by great crystallizing power. It formed sparingly soluble prisms from 95 per cent alcohol which did not exhibit a definite melting point. It slowly softened above 215° but the mass did not become clear until 247°.

4.011 mg. substance: 3.119 mg. H_2O, 10.868 mg. CO_2.
$C_{23}H_{32}O_4$. Calculated. C 74.14, H 8.66
Found. " 73.89, " 8.70

Ethylal of β-Hexahydrooxidodianhydrostrophanthidin—The preceding unsaturated ethylal in ethyl alcoholic solution rapidly absorbed 1 mol of hydrogen when either palladium or platinum catalysts were used. The saturated substance readily crystallized from alcohol as needles which melted at 131–132°.

$[\alpha]_D^{24} = +13.4°$ (c = 1.466 in alcohol).
3.917 mg. substance: 3.291 mg. H_2O, 10.681 mg. CO_2.
$C_{25}H_{38}O_4$. Calculated. C 74.57, H 9.52
Found. " 74.36, " 9.40

This substance resembles the α-isomer in general properties. On heating with alcoholic hydrogen chloride it is also isomerized to a mixture of substances which were not investigated.

β-Hexahydrodianhydrostrophanthidin—The above ethylal was warmed in 10 parts of 50 per cent acetic acid until solution was complete. On dilution and chilling the free hydroxyaldehyde separated. When recrystallized from methyl alcohol it formed needles which melted at 155–156° after preliminary softening.

$[\alpha]_D^{20} = +20°$ (c = 0.705 in chloroform).
3.687 mg. substance: 2.972 mg. H_2O, 9.955 mg. CO_2.
$C_{23}H_{34}O_4$. Calculated. C 73.74, H 9.15
Found. " 73.63, " 9.02

This substance reacts in either the free aldehydic or lactol form.

The *oxime* was obtained in the usual manner and crystallized from alcohol as needles which melted at 262° after preliminary softening.

4.270 mg. substance: 3.410 mg. H_2O, 11.102 mg. CO_2.
6.350 " " : 0.217 cc. N (23°, 755 mm.).
$C_{23}H_{35}O_4N$. Calculated. C 70.90, H 9.06, N 3.60
Found. " 70.90, " 8.93, " 3.90

Like the α-isomer, β-hexahydrodianhydrostrophanthidin is oxidized in the lactol form by Kiliani solution to a *hexahydrodilactone.* This appeared to be also a mixture of isomers in which a sparingly soluble high melting form seemed to predominate. After repeated recrystallization from acetone it formed fine needles which melted at 268–269°. It appeared to be identical with the previously described high melting dilactone obtained as a minor fraction of the oxidation product of α-hexahydrodianhydrostrophanthidin.

$[\alpha]_D^{24} = +13.6°$ (c = 0.825 in chloroform).
3.943 mg. substance: 3.060 mg. H_2O, 10.770 mg. CO_2.
$C_{23}H_{32}O_4$. Calculated. C 74.14, H 8.67
Found. " 74.50, " 8.68

Lower melting material which was recovered from the mother liquors was not further investigated.

Benzoyl-β-Hexahydrodianhydrostrophanthidin—Acylation was accomplished with benzoyl chloride in pyridine solution. The product formed sparingly soluble lustrous leaflets from 95 per cent alcohol, which melted at 209–211°.

2.594 mg. substance: 1.842 mg. H_2O, 7.137 mg. CO_2.
$C_{30}H_{38}O_5$. Calculated. C 75.27, H 8.01
Found. " 75.03, " 7.95

This substance is a derivative of the aldehydic form since it yields an *oxime*. For a complete reaction it was necessary to reflux an alcoholic solution of the reactants for about 5 hours. The oxime formed short prisms from alcohol which melted at 252°.

4.685 mg. substance: 3.255 mg. H_2O, 12.550 mg. CO_2.
5.150 " " : 0.137 cc. N (24°, 750.5 mm.).
$C_{30}H_{39}O_5N$. Calculated. C 72.97, H 7.97, N 2.84
Found. " 73.05, " 7.76
" " 3.02

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THE HYDROGENATION OF UNSATURATED LACTONES TO DESOXY ACIDS. II

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In a previous communication[1] it has been shown that in the case of the unsaturated lactones derived from the enolic forms of certain γ- and δ-keto acids in which the double bond is at the point of lactonization, hydrogenation proceeds with the absorption of 2 mols of H_2 to the saturated desoxy acid. A question which required further investigation, however, was the apparent inconsistency shown in the fact that the strophanthidin group of cardiac aglucones which are $\Delta^{\beta,\gamma}$-unsaturated γ-lactones appeared on hydrogenation to yield only neutral dihydro derivatives. In the case of strophanthidin[2] itself under very careful conditions and by the use of a very active catalyst (Adams and Shriner) the difficult additional step of reduction of the aldehyde group to the primary alcohol could be accomplished but without the formation of an appreciable amount of acid. In order to explain the failure of these aglucones to form desoxy acids it became advisable to extend somewhat the study of simpler unsaturated lactones in order to include certain features associated with the unsaturated lactone group of these aglucones. These aglucones are γ-lactones of the enolic form of aldehydo acids. Further, they are substituted in the β-position. We have, therefore, chosen for further study under exactly comparable conditions a few accessible substances which would indicate the effect on previously studied substances first of β-substitution and then of changing from the lactone of a keto acid to that of an aldehydo acid.

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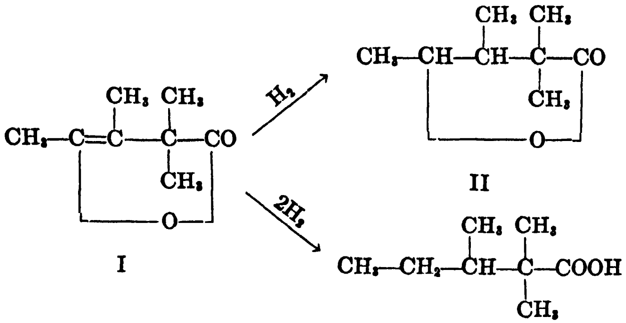
[1] Jacobs, W. A., and Scott, A. B., *J. Biol. Chem.*, **87**, 601 (1930).

[2] Jacobs, W. A., *J. Biol. Chem.*, **88**, 528 (1930).

Whereas α,α-dimethyl-$\Delta^{\beta,\gamma}$-angelicalactone[1] was previously shown to go completely to the desoxy acid, α, α, β-trimethyl-$\Delta^{\beta,\gamma}$-angelicalactone (I) yielded roughly only 50 per cent of *α, α, β-trimethylvaleric acid* (III). The other reaction product was *α, α,-β-trimethyl-γ-valerolactone* (II). A similar result was obtained on hydrogenating β-methyl-$\Delta^{\beta,\gamma}$-angelicalactone (IV). $\Delta^{\beta,\gamma}$-Angeli-



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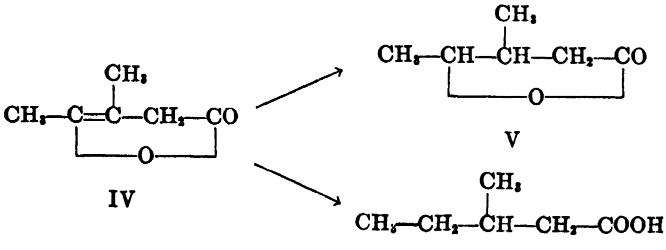

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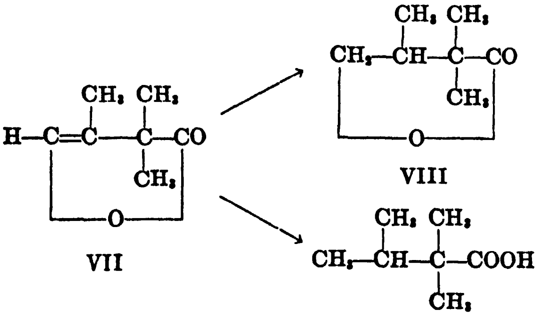

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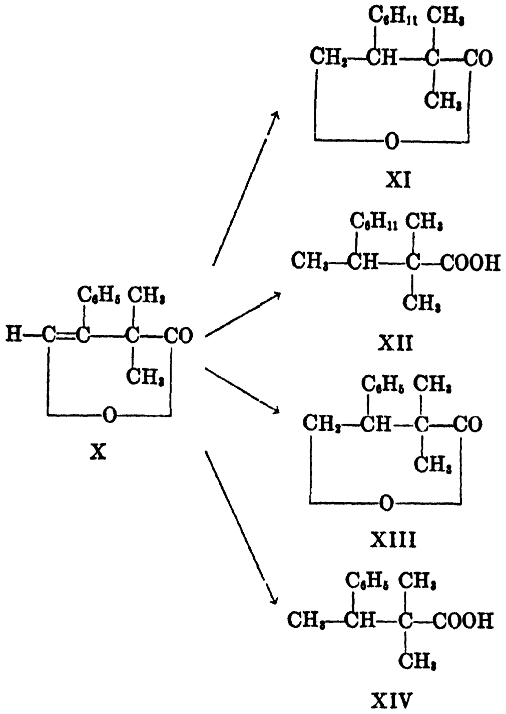

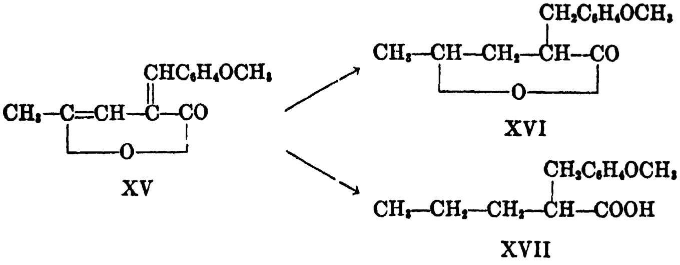

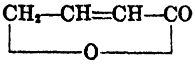

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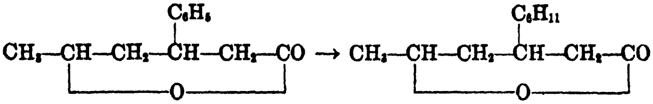

XIX XX

calactone[3] goes completely to valeric acid. In the case of its β-methyl derivative 70 per cent, at the most, was converted into β-methylvaleric acid (VI). A neutral fraction of β-methyl-γ-valerolactone (V) was also obtained. The experience with these two substances thus indicates that β-substitution in $\Delta^{\beta,\gamma}$-γ-lactones is a factor which diminishes hydrogenation to desoxy acids.

Opportunity to test the influence of a change from a γ-keto acid lactone to a γ-aldehydo acid lactone was presented by the hydrogenation of α, α, β-trimethyl-$\Delta^{\beta,\gamma}$-butenolide (VII). In this case the major reaction product was α, α, β-trimethylbutyrolactone (VIII), while only 21 per cent was converted to α, α, β-trimethylbutyric acid (IX). The acid formation here was, therefore, less than half of that observed in the case of the analogous ketolactone.

It might be suggested that the influence of the β-methyl group is only an apparent one and that the observed effect is really due to a partial shift of the double bond to a position between the β-carbon atom and the carbon atom of this methyl group, thus changing the latter to methylene. In such a case a saturated lactone would result perhaps without desoxy acid formation. This explanation, however, is very improbable, especially in view of our experience with another substance where such a shift has been excluded. This substance was α, α-dimethyl-β-phenyl-$\Delta^{\beta,\gamma}$-butenolide (X). On exhaustive hydrogenation with complete reduction of the aromatic nucleus only about 25 per cent was reduced to α, *α-dimethyl-β-cyclohexylbutyric acid* (XII). The major reaction product was α, *α-dimethyl-β-cyclohexylbutyrolactone* (XI). It is practically excluded that the relatively low acid formation in this case was caused by the shifting of double bonds during the hydrogenation of the aromatic nucleus itself. This was shown by the fact that when the reaction was stopped at approximately the 2 mol stage the same proportion of desoxy acid and neutral lactone resulted. These substances were respectively *α, α-dimethyl-β-phenylbutyric acid* (XIV) and *α, α-dimethyl-β-phenylbutyrolactone* (XIII).

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[3] Since our first communication Professor Asahina has kindly brought to our attention his earlier paper (Asahina, Y., and Fujita, A., *Acta Phytochimica*, **1**, 1 (1922)) containing observations which appear to have remained generally unknown. In their studies on the structure of anemonin it was noted that α-angelicalactone yields *n*-valeric acid on catalytic hydrogenation. This observation was made to explain the similar catalytic reduction of dihydroanemonin to sebacic acid. These workers appear to be the first to have noted the catalytic reduction of unsaturated lactones to desoxy acids.

From these results the behavior of the cardiac aglucones as lactones of β-substituted γ-aldehydo acids no longer represents an inconsistency. The failure in these cases of any appreciable acid formation is apparently due to the added effect of the complexity of the portion of the molecule which is here the β-substituent. The β-phenyl substituted lactones which Mannich and Butz[4] and the writers have found to be completely hydrogenated to desoxy acids are δ-lactones. The latter as a group are more readily opened than γ-lactones. A greater tendency toward reductive cleavage in the case of δ-lactones should tend to counteract any inhibiting effect of β-substitution. The complete reduction of anhydro-γ-isogitoxigenic methyl ester to the desoxy acid[5] in spite of the fact that its unsaturated lactone group is β-substituted may be explained by the fact that it is a δ-lactone. A similar instance has been recently[6] noted in the case of the unsaturated δ-lactone anhydride, $C_{21}H_{24}O_5$, obtained from duodephanthondiacid.

In the course of this work the opportunity was presented to include a few additional unsaturated lactones in the study. In the case of one of these, α-anisal-$\Delta^{\beta,\gamma}$-angelicalactone (XV), the hydrogenation was arbitrarily interrupted after the absorption of 2.4 mols of H_2 (where a definite change in the absorption rate had occurred) to avoid the complication of reduction of the aromatic nucleus. Contrary to α, α-dimethylangelicalactone,[1] this substance gave mostly *α-anisyl-γ-valerolactone* (XVI) and only 17 per cent of the desoxy acid *α-anisylvaleric acid* (XVII). In this case the presence of conjugated double bonds unquestionably influenced the course of the reaction. The predominating reaction can be pictured as 1–4 addition to the two double bonds with the intermediate formation of α-anisyl-$\Delta^{\alpha,\beta}$-angelicalactone which was then reduced to the saturated lactone. With the hope of studying the hydrogenation of the simplest γ-aldehydo acid lactone, $\Delta^{\beta,\gamma}$-butenolide (XVIII) was employed since the $\Delta^{\beta,\gamma}$-isomer was not available. On hydrogenation this lactone gave roughly 90 per cent of butyrolactone and 10 per cent of butyric acid. A small amount of $\Delta^{\beta,\gamma}$-butenolide, already present or possibly formed during the hydrogenation, was the probable source of this butyric acid.

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[4] Mannich, C., and Butz, A., *Ber. chem. Ges.*, **62**, 461 (1929).

[5] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **86**, 203 (1930).

[6] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **92**, 338 (1931).

In our previous paper it was shown that the lactone of δ-hydroxy-β-phenyl-$\Delta^{\gamma,\delta}$-hexenoic acid goes completely to the desoxy acid without the apparent formation of an intermediate saturated lactone, β-phenyl-δ-caprolactone (XIX). This was at variance with the results reported by Mannich and Butz[4] in the case of the lactone of δ-hydroxy-β, δ-diphenyl-$\Delta^{\gamma,\delta}$-pentenoic acid and the analogous β-methylenedioxyphenyl derivative. Our conclusion has been more recently substantiated by an examination of the behavior of β-phenyl-δ-caprolactone itself. On exhaustive hydrogenation, which involved the aromatic nucleus, this substance yielded but a negligible amount of acid. Practically all was a neutral mixture of stereoisomeric forms of β-cyclohexyl-δ-caprolactone (XX).

EXPERIMENTAL

In the following hydrogenation experiments a constant procedure was used. The solvent was ethyl alcohol which had been distilled three times through an all glass apparatus. The catalyst was the platinum oxide catalyst of Adams and Shriner[7] prepared, however, with the modification of longer heating recommended by Sigmund.[8] In all experiments the same lot of catalyst was used. Hydrogenation was carried out at 20–25° and at atmospheric pressure. Gas volumes given are all for the dry gas at normal temperature and pressure and are corrected for the calculated absorption due to the reduction of the oxide catalyst.

Hydrogenation of α, α, β-Trimethyl-$\Delta^{\beta,\gamma}$-Angelicalactone—Bardhan's method[9] for the preparation of this lactone, which consisted in the thermal decomposition of β, γ-dibromo-α, α, β-trimethylvaleric acid, was followed. After several fractionations material was collected of b.p.$_{13}$ 80–85°, which still gave a positive halogen test. For purification this material was boiled for 1 hour with 4 parts of quinoline. The mixture was distilled at about 20 mm. until the distillate became red. After the latter was treated with dilute H_2SO_4 in excess, the mixture was extracted with ether. The ether solution after being washed with sodium carbonate solution yielded the halogen-free lactone of b.p.$_{13}$ 79–80° (Bardhan gives b.p.$_{13}$ 80°).

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[7] Adams, R., and Shriner, R. L., *J. Am. Chem. Soc.*, **45**, 2171 (1923).

[8] Sigmund, F., *Sitzungsber. Akad. Wissensch. Wien. Math.-natur. Klasse, 2. Abt.*, **138**, 611 (1929).

[9] Bardhan, J. C., *J. Chem. Soc.*, 2616 (1928).

3.84 gm. of the lactone were hydrogenated with an initial 60 mg. of catalyst. Since the absorption for some undetermined reason was slow, occasional additional amounts of catalyst were added, making a total of 0.48 gm. After 76 hours, absorption of hydrogen stopped at a total of 965 cc. Calculated for 1 mol of H_2, 615 cc. This indicated that 57 per cent was reduced to desoxy acid.

Direct titration for free acid against phenolphthalein required 13.2 cc. of N NaOH. An excess of alkali was then added to the solution which after being refluxed for 3 hours was titrated back. An additional 16.0 cc. of N NaOH were required, or a total of 29.2 cc. Calculated, 27.7 cc. From the direct titration 48 per cent of the lactone was converted into desoxy acid.

The above alkaline solution was concentrated and after being acidified with an excess of H_2SO_4 it was boiled to relactonize hydroxy acid. An ether extract was carefully shaken out with dilute sodium carbonate solution in order to remove trimethylvaleric acid. After the ether solution was washed with saturated NaCl solution it was dried and concentrated. The neutral residue which weighed 1.77 gm. was trimethyl-γ-valerolactone and showed a b.p.$_{33}$ 121–123°.

4.967 mg. substance: 4.352 mg. H_2O, 12.275 mg. CO_2.

$C_8H_{14}O_2$. Calculated. C 67.56, H 9.92

Found. " 67.40, " 9.80

The above Na_2CO_3 solution of trimethylvaleric acid was reacidified and extracted with ether. The ether residue on fractionation boiled at 137–138° at 43 mm.

5.940 mg. substance: 5.885 mg. H_2O, 14.490 mg. CO_2.

$C_8H_{16}O_2$. Calculated. C 66.62, H 11.17

Found. " 66.57, " 11.09

In a second experiment complete absorption of hydrogen was accomplished within 3 hours. In this case the excessive hydrogen absorption over 1 mol was 59 per cent. The amount of free acid found by titration was 52 per cent.

Hydrogenation of β-Methyl-$\Delta^{\beta,\gamma}$-Angelicalactone—This lactone was prepared from β-methyllevulinic acid by the method used by Wolff[10] in the case of levulinic acid. The keto acid was slowly distilled during 3 hours at ordinary pressure and the distillate was taken up in ether. The dry ethereal solution was concentrated and carefully fractionated. In this manner β-methyl-$\Delta^{\beta,\gamma}$-angelicalactone, b.p.$_{13}$ 83–86°, was prepared (Pauly, Gilmour, and Will[11] give b.p.$_{13}$ 87–89°). An alcoholic solution of 1.02 gm. of this lactone with 0.05 gm. of catalyst absorbed 309 cc. of H_2 in 33 minutes. Calculated for 1 mol of H_2, 204 cc. This indicated that 51 per cent of desoxy acid was formed. Direct titration of free acid required 64.0 cc. of N NaOH, or 70 per cent of free acid. A second portion of 1.07 gm. with 0.06 gm. of catalyst absorbed 323 cc. of H_2 in 36 minutes. Calculated for 1 mol of H_2, 214 cc. Direct titration of free acid required 67 cc. of N NaOH. This indicated again 51 and 70 per cent of desoxy acid by the two measurements. For this wide divergence of the hydrogen absorption and the direct titration we have no explanation.

The two portions were combined at this point and required 5.7 cc. of N NaOH for saponification. Total observed alkali equivalent, 18.8 cc. of N NaOH. Calculated, 18.7 cc. The neutral and acidic products were isolated as in the previous case.

The crude neutral fraction which consisted of β-methylvalerolactone was about 0.35 gm. and distilled roughly at 60–70° and 14 mm.

5.144 mg. substance: 4.110 mg. H_2O, 11.935 mg. CO_2.

$C_6H_{10}O_2$. Calculated.	C 63.10,	H 8.82
Found.	" 63.28,	" 8.94

The acid fraction gave 1.5 gm. of crude β-methylvaleric acid, which on distillation showed a b.p.$_{758}$ 197–198° (corrected). The silver salt was prepared for further characterization.

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[10] Wolff, L., *Ann. Chem.*, **229**, 251 (1885).

[11] Pauly, H., Gilmour, R., and Will, G., *Ann. Chem.*, **403**, 152 (1914).

6.631 mg. substance: 2.930 mg. H_2O, 7.840 mg. CO_2, 3.220 mg. Ag.

$C_6H_{11}O_3Ag$.	Calculated.	C 32.24,	H 4.98,	Ag 48.39
	Found.	" 32.25,	" 4.94,	" 48.56

Hydrogenation of α, α, β-Trimethyl-$\Delta^{\beta,\gamma}$-Butenolide—The lactone was prepared by the procedure of Courtot[12] and Blaise and Courtot,[13] except that the final removal of halogen was accomplished by boiling with quinoline. This treatment gave halogen-free lactone of b.p.$_{12}$ 60–63° (Blaise and Courtot give b.p.$_{12}$ 65°).

1.43 gm. of this lactone were hydrogenated with 0.1 gm. of catalyst. At the end of 73 minutes absorption stopped at 305 cc. of hydrogen. Calculated for 1 mol of H_2, 254 cc. The excess indicated formation of 20 per cent of desoxy acid. Direct titration of free acid required 2.4 cc. of N NaOH, or 21 per cent of acid formation. For saponification there was required an additional 8.1 cc. of N NaOH, or a total of 10.5 cc. Calculated, 11.3 cc.

After relactonization of the above alkaline solution the neutral and acid fractions were separated as usual. The neutral fraction of α, α, β-trimethylbutyrolactone (0.9 gm.) was distilled at atmospheric pressure and crystallized at room temperature. The b.p. of 204–206° was obtained. (Noyes[14] gives b.p. 211–212°). On redistillation it showed a b.p.$_{14}$ 92–94°.

7.174 mg. substance: 6.132 mg. H_2O, 17.217 mg. CO_2.

$C_7H_{12}O_2$.	Calculated.	C 65.58,	H 9.45
	Found.	" 65.45,	" 9.56

The crude acid fraction (0.3 gm.) of α, α, β-trimethylbutyric acid of b.p.$_{13}$ 104–105° gave a colorless oil which crystallized readily in the receiver (Haller and Bauer[15] give b.p.$_{13}$ 104–105°).

5.461 mg. substance: 5.230 mg. H_2O, 12.995 mg. CO_2.

$C_7H_{14}O_2$.	Calculated.	C 64.57,	H 10.84
	Found.	" 64.90,	" 10.71

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[12] Courtot, A., *Bull. Soc. Chim.*, series 3, 35, 298 (1906).

[13] Blaise, E. E., and Courtot, A., *Bull. Soc. Chim.*, series 3, 35, 995 (1906).

[14] Noyes, W. A., *Am. Chem. J.*, 33, 362 (1905).

[15] Haller, A., and Bauer, E., *Compt. rend. Acad.*, 149, 6 (1909).

The silver salt formed slender needles from water.

5.034 mg. substance: 2.458 mg. H_2O, 6.425 mg. CO_2, 2.287 mg. Ag.
$C_7H_{11}O_2Ag$. Calculated. C 35.43, H 5.45, Ag 45.55
Found. " 34.93, " 5.45, " 45.27

Hydrogenation of α, α-Dimethyl-β-Phenyl-$\Delta^{\beta,\gamma}$-Butenolide—This lactone was prepared by the method of Blaise and Courtot.[16] An alcoholic solution of 0.56 gm. of this lactone was hydrogenated with 50 mg. of catalyst. The experiment was interrupted at the end of 202 minutes after 134 cc. had been absorbed. Calculated for 2 mols of H_2, 134 cc. Direct titration of free acid required 0.73 cc. of N NaOH, or 24 per cent, while after saponification an additional 2.1 cc. of N NaOH were consumed, or a total of 2.83 cc. Calculated, 2.98 cc.

The neutral fraction recovered after relactonization was obtained from dry ether as leaflets which melted at 91–92°. The substance was moderately soluble in ethyl alcohol and petrolic ether and readily soluble in ether, acetone, and benzene. The analytical figures agreed with α, α-dimethyl-β-phenylbutyrolactone.

3.811 mg. substance: 2.590 mg. H_2O, 10.605 mg. CO_2.
$C_{12}H_{14}O_2$. Calculated. C 75.75, H 7.42
Found. " 75.88, " 7.60

The acid fraction was obtained as an oil which readily crystallized. After recrystallization from dilute acetone the substance melted at 61–62° and was very soluble in ether, acetone, benzene, and petrolic ether. The analytical figures agreed with α, α-dimethyl-β-phenylbutyric acid.

3.510 mg. substance: 2.640 mg. H_2O, 9.668 mg. CO_2.
$C_{12}H_{16}O_2$. Calculated. C 74.96, H 8.38
Found. " 75.12, " 8.41

In a second experiment the hydrogenation was allowed to go to completion involving the aromatic nucleus. This resulted in the formation of cyclohexyl compounds corresponding to the products of the first experiment. 1.22 gm. of the unsaturated lactone and

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[16] Blaise, E. E., and Courtot, A., *Bull. Soc. Chim.*, series 3, **35**, 1002 (1906).

0.14 gm. of catalyst were used. The absorption of H_2 was 639 cc. at the end of 23 hours. Calculated for 4 mols of H_2, 580 cc. This excess indicated 41 per cent of acid formation. This was considerably higher than that shown by the direct titration for free acid, *i.e.* 1.62 cc. of N NaOH, or 25 per cent. Saponification required an additional 4.0 cc. of N NaOH, or a total of 5.62 cc. Calculated, 6.49 cc.

After relactonization 1.06 gm. of α, α-dimethyl-β-cyclohexylbutyrolactone were obtained. It formed large plates from ether which melted at 51–52°.

4.090 mg. substance: 3.730 mg. H_2O, 11.00 mg. CO_2.

$C_{12}H_{20}O_2$.	Calculated.	C 73.39, H 10.30
	Found.	" 73.35, " 10.21

The acid fraction of α,α-dimethyl-β-cyclohexylbutyric acid crystallized from dilute acetone as irregular plates which melted at 39–40°.

3.670 mg. substance: 3.682 mg. H_2O, 9.753 mg. CO_2.

$C_{12}H_{22}O_2$.	Calculated.	C 72.65, H 11.20
	Found.	" 72.48, " 11.23

Hydrogenation of α-Anisal-$\Delta^{\beta,\gamma}$-Angelicalactone—The lactone was prepared by the method of Thiele.[17] For 4.4 gm. of this lactone suspended in alcoholic solution 0.15 gm. of catalyst was used. The hydrogenation was interrupted after 3 hours when there was a definite slowing up of the rate of absorption. This was done to avoid the possible complication of hydrogenation of the aromatic nucleus. The observed hydrogen absorption was 1087 cc. Calculated for 2 mols of H_2, 912 cc. Direct titration of free acid required 3.5 cc. of N NaOH, while saponification with an excess of alkali required an additional 19.7 cc. of N NaOH, or a total of 23.2 cc. Calculated, 20.4 cc.

2.6 gm. of neutral material were recovered after relactonization and proved to be α-anisyl-γ-valerolactone. On recrystallization from petrolic ether it formed glistening plates which melted at 60–61° and were soluble in the usual solvents. The saturated character of the substance was shown by its behavior toward bromine and permanganate.

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[17] Thiele, J., Tischbein, R., and Lossow, E., *Ann. Chem.*, **319**, 185 (1901).

4.098 mg. substance: 2.595 mg. H_2O, 10.655 mg. CO_2.
$C_{13}H_{16}O_3$. Calculated. C 70.88, H 7.31
Found. " 70.91, " 7.09

0.75 gm. of an acid fraction was recovered and proved to be α-anisylvaleric acid. On fractionation it gave a rough boiling point of 180–185° at 7 mm. and slowly crystallized. The crystalline material melted at 50–52° and was soluble in the usual organic solvents.

4.025 mg. substance: 2.953 mg. H_2O, 10.390 mg. CO_2.
$C_{13}H_{18}O_3$. Calculated. C 70.24, H 8.15
Found. " 70.40, " 8.21

Hydrogenation of $\Delta^{\alpha,\beta}$-Butenolide—This lactone has previously been prepared by Lespieau,[18] and Lespieau's lactone has been nearly quantitatively oxidized to erythronic lactone by Braun.[19] Lespieau's preparation was made by thermal decomposition of 3,4-dichlorobutyric acid. However, 3-chlorocrotonic acid could be readily prepared by the recent method of Braun,[20] so $\Delta^{\alpha,\beta}$-butenolide in this investigation was prepared by thermal decomposition of 3-chlorocrotonic acid as follows:

39 gm. of 3-chlorocrotonic acid were heated for 30 minutes in a metal bath kept at 200–220° under a reflux. A current of dry air was swept through the apparatus during the heating in order to remove HCl gas which was vigorously evolved during the first 10 minutes. The residual brown syrup was dissolved in ether. The solution was washed with sodium bicarbonate solution and dried. Distillation gave 5.8 gm. of butenolide of b.p.$_{13}$ 94–95° (Lespieau[18] gives b.p.$_{13}$ 95–96°). The material in dilute solution did not reduce ammoniacal silver nitrate solution at ordinary temperature but upon the addition of dilute NaOH the reduction was prompt. A positive nitroprusside test was obtained.

[18] Lespieau, R., *Bull. Soc. Chim.*, series 3, **33**, 466 (1905).
[19] Braun, G., *J. Am. Chem. Soc.*, **51**, 235 (1929).
[20] Braun, G., *J. Am. Chem. Soc.*, **52**, 3167 (1930).

6.785 mg. substance: 3.030 mg. H_2O, 14.190 mg. CO_2.
$C_4H_4O_2$. Calculated. C 57.14, H 4.76
Found. " 57.04, " 4.99

For the hydrogenation 4.55 gm. of the lactone and 60 mg. of catalyst were used. 1321 cc. of H_2 were absorbed during 70 minutes. Calculated for 1 mol of H_2, 1214 cc. After filtration from catalyst the solution smelled of butyric acid. Direct titration of free acid required 6.8 cc. of N NaOH, or 13 per cent of acid formation. Saponification with an excess of alkali required an additional 50.0 cc. of N NaOH, or a total of 56.8 cc. Calculated, 54.2 cc.

After relactonization 2.75 gm. of crude butyrolactone were recovered. This boiled at 201°.

5.648 mg. substance: 3.592 mg. H_2O, 11.610 mg. CO_2.
$C_4H_6O_2$. Calculated. C 55.81, H 6.98
Found. " 56.06, " 7.08

Hydrogenation of β-Phenyl-δ-Caprolactone—This lactone was readily prepared by reduction of β-phenyl-δ-ketocaproic acid in alcoholic solution with sodium. A refluxing solution of 12 gm. of the keto acid in 50 cc. of absolute alcohol was treated with 7.2 gm. of sodium during 20 minutes. During a further 20 minutes 60 cc. of alcohol were added to decompose all of the sodium. After dilution the alcohol was removed under diminished pressure. The residual aqueous solution was relactonized with an excess of H_2SO_4. The ether extract yielded 4.6 gm. of lactone of b.p.$_{19}$ 197–200°.

4.240 mg. substance: 2.950 mg. H_2O, 11.775 mg. CO_2.
$C_{12}H_{14}O_2$. Calculated. C 75.75, H 7.42
Found. " 75.74, " 7.78

3.2 gm. of the lactone with 0.15 gm. of catalyst absorbed 1222 cc. of H_2 in 70 hours. Calculated for 3 mols of H_2, 1131 cc. Direct titration required only 0.46 cc. of N NaOH, while saponification by boiling several hours with an excess of alkali required 13.90 cc. of N NaOH, or a total of 14.36 cc. of N NaOH. Calculated, 16.8 cc. of N NaOH.

After relactonization the acid portion recovered was negligible. The crude neutral fraction of β-cyclohexyl-δ-caprolactone weighed

2.9 gm. This material which was unquestionably a mixture of stereoisomers partially crystallized at room temperature. It still gave a mixture of liquid and crystals when distilled, and did not completely crystallize after long standing. This material gave a b.p.$_{20}$ 194–198°.

8.238 mg. substance: 7.580 mg. H_2O, 22.197 mg. CO_2.
$C_{12}H_{20}O_2$. Calculated. C 73.39, H 10.29
Found. " 73.49, " 10.31

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A NOTE ON A MODIFICATION OF PREGL'S MICRO-METHOXYL APPARATUS

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Considerable experience in this Laboratory with Pregl's[1] micro apparatus for the estimation of methoxyl has shown certain difficulties. The operation requires very close attention on account of the marked bumping of the contents of the decomposition flask and the consequent

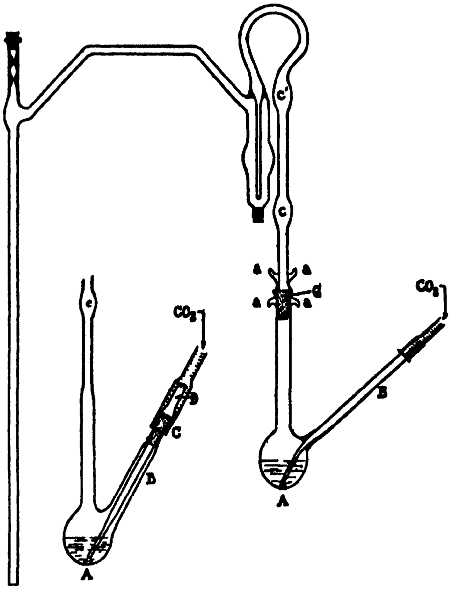

Fig. 2. Fig. 1

One-third actual size

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[1] F. Pregl, "Quantitative Organic Microanalysis," second English edition translated from the third German edition by E. Fyleman, P. Blakiston's Son & Co., Inc., Philadelphia, Pa., 1930, p. 181.

danger of the sucking back of the washing solution from the washer and that of the silver nitrate solution from the receiver. To avoid these difficulties the following modifications of Pregl's apparatus have been designed and placed in use.

1. This modification (Fig. 1) is a two-piece apparatus in which the two parts are connected by means of a ground-glass joint at G. The upright outlet tube is provided with two bulbs c,c′ to condense any hydriodic acid carried along by the carbon dioxide stream. The carbon dioxide is led into flask A through the tube B which is sealed on the side of the flask and drawn into a capillary reaching almost to the bottom of the latter. In the procedure the apparatus is charged through the opening G. The ground joint is then moistened with a drop of hydriodic acid and replaced. Two rubber bands are attached to the hooks a, in order to hold the two parts tightly and the tube B is connected with the carbon dioxide generator. The rest of the procedure is as described by Pregl.

2. In this one-piece Pregl's apparatus (Fig. 2) the carbon dioxide is led into the decomposition flask A through the tube D drawn into a capillary and entering the side arm B of the flask. This tube fits tightly at the outer end of the side arm by means of a ground-glass joint C. After the apparatus has been charged through the side arm B, the inside of the latter and the ground joint C are moistened with hydriodic acid and the tube D inserted. Then the rubber tube leading to the carbon dioxide generator is slipped over the end of the glass tube D and over the ground-glass joint. There is no retention of alkyl iodide in the side arm on account of the presence of hydriodic acid.

Numerous methoxyl determinations with either modification 1 or 2 have demonstrated that boiling is smooth, bumping does not occur, accurate results are obtained and almost no attention to the operation is necessary after the initial adjustment of the carbon dioxide stream and the microburner.

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THE PRESENCE OF A GELATIN-LIQUEFYING ENZYME IN CRUDE PEPSIN PREPARATIONS

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INTRODUCTION

In the course of a study of the fractionation of crude pepsin preparations it was found that amorphous precipitates could be obtained which were extremely active with respect to liquefaction of gelatin (1). In the study of the properties of the crystalline protein isolated from crude pepsin preparations it was also noted occasionally that a small amount of amorphous material was obtained which had this strong gelatin-liquefying power. When these preparations were crystallized the abnormal activity disappeared, and the crystalline material had the same activity as the usual crystalline pepsin. It seemed possible therefore that this activity was due to the physical state of the precipitate, a possibility which has frequently been suggested in the literature. A more careful study of this material, however, showed that this was not the case, but that the activity was due to a distinct enzyme, "gelatinase," which is extremely active with respect to the liquefaction of gelatin. This "gelatinase" is present in very small quantities in the original material and is completely removed from the crystalline pepsin only with considerable difficulty. The conclusion that the abnormal activity is due to this "gelatinase" depends upon the fact that the abnormally active material cannot be obtained from crystalline pepsin which has been crystallized four or five times. In addition it was found that the activity of the crystalline pepsin could be destroyed in slightly alkaline solution while the gelatin liquefying power of the amorphous preparations was only slightly reduced. This accounts for the occasional abnormal results which were obtained in the study of the alkali-inactivation of crystalline pepsin.

A method has been worked out for the purification of the "gelatinase" and a small amount of protein material has been obtained which was about 450 times as active as the crystalline pepsin as measured by the liquefaction of gelatin, or about 1,000 times as active as the crude pepsin preparation. The activity of this material could not be increased by any method tried, and solubility measurements gave an indication that it might be a pure protein. Owing to lack of material, however, no conclusive results were obtained as to the final purity of the preparation.

A study has been made of the activity of this material in effecting the hydrolysis of gelatin, casein, egg albumin, and edestin as measured by the change of viscosity, increase in formol titration, and production of non-protein nitrogen. The activities of the crude pepsin and crystalline pepsin have also been determined in the same way. The "gelatinase" is considerably less active than the crystalline pepsin in all respects except in the hydrolysis of gelatin. It is three or four times as active as crystalline pepsin in the hydrolysis of gelatin as measured by the formol titration, and 450 times as active as measured by the change of viscosity. It is possible that the activity on proteins other than gelatin is due to small amounts of the crystalline pepsin present in the "gelatinase" preparations. On the other hand, crystalline pepsin preparations which have apparently been entirely freed from the "gelatinase" still possess the power of digesting gelatin.

EXPERIMENTAL RESULTS

Separation from Crystalline Pepsin.—The abnormal activity with gelatin was originally noted when the protein fraction from crude pepsin preparations was dissolved with dilute alkali and partially precipitated with acid. When the precipitate obtained in this way was fractionated by repeated partial precipitation the gelatin-liquefying power increased, and this process could be apparently repeated indefinitely. The proteolytic power as shown with other proteins also increased at first, remained constant after four or five precipitations, and eventually decreased. If the same process be carried out with crystalline pepsin which has been crystallized once or twice, a small quantity of abnormally active amorphous material can be obtained after five or ten partial precipitations. If, however, the crystalline pepsin has been recrystallized four or five times very little or none of the abnormally active material can be obtained.

Separation by Alkali Inactivation.—If solutions of crude pepsin or of once or twice crystallized pepsin are brought to pH 9.5 or pH 10 for a few minutes and then acidified, the solution still liquefies gelatin more or less rapidly, while if the crystalline pepsin has been recrystallized several times the gelatin-liquefying power is completely lost at pH 9.5 in a very short time. This procedure serves therefore as a sensitive test for the presence of the "gelatinase." A comparison of the loss of activity of four-times recrystallized pepsin and of a "gelatinase" fraction is shown in Fig. 1. The figure shows that the loss in activity of the crystalline pepsin as measured by loss in gelatin-liquefying power or by the hydrolysis of casein is about the same and is practically complete at pH 9. The "gelatinase" fraction, on the other hand, is less than half inactivated under the same conditions and retains a considerable part of its activity at pH 10.

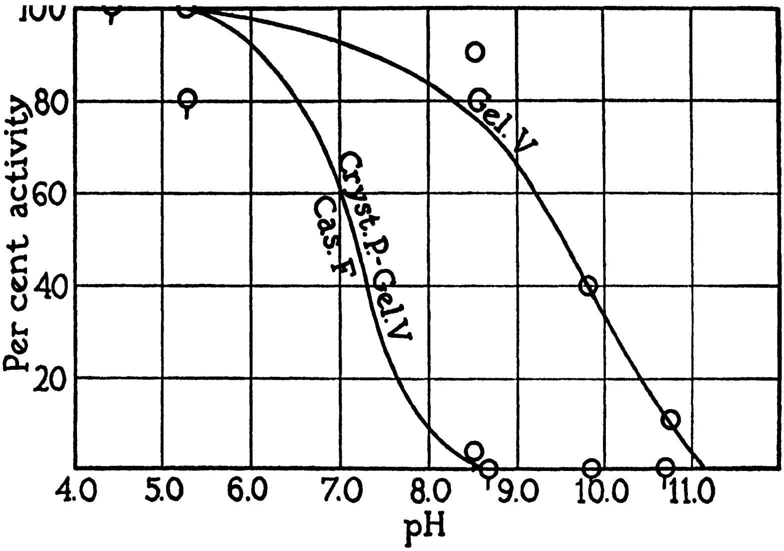

FIG. 1. Loss of activity of crystalline pepsin and crude "gelatinase" at different pH, as measured by the liquefaction of gelatin and by the digestion of casein.

Preparation of "Gelatinase."—The repeated precipitation at pH 3, mentioned above, leads to increasingly active preparations; but it soon became evident that no appreciable amount of material could ever be obtained in this way, owing to the cumulative losses in the

TABLE I

Preparation of "Gelatinase"

	Fraction no.
1,000 gm. Parke, Davis pepsin U. S. P. 1:10,000 + 800 ml. H_2O + 800 ml. M/1 H_2SO_4 + 1,600 ml. saturated $MgSO_4$.	
Filter. Filtrate discard.	
Ppt.	1
Ppt. 1, stir with ⅓ volume water and M/2 NaOH stirred in until clear solution (pH <5.0). N/2 H_2SO_4 stirred until pH = 3.0. Stand at 6°C. for 18 hrs.	
Filter. Filtrate.	F_2
Stand at 6°C., 4 to 6 days, ppt. forms. Filter and discard filtrate.	
Ppt.	P_2 *a*
Ppt. Dissolve at 37°C. with ⅓ volume H_2O and M/2 NaOH. Crystallize.	
Filter. Ppt. Crystalline pepsin, recrystallize 3 times	3 C
" 4 "	4 C
Filtrate. Add ⅓ to ½ volume saturated Na_2SO_4.	
Filter. Filtrate.	F_3
Ppt., combine with ppt. P_{2a}, dissolve with M/10 pH 5.0 citrate buffer and dilute to 5.0 mg. N/ml. Precipitate by addition ⅓ to ½ volume saturated Na_2SO_4, filter and repeat precipitation until ppt. has activity of about $5 \times 10^4\ [\text{PU}]^{\text{gel. }5}_{\text{gm. N.}}$	P_4
(Combined filtrates from this process may be titrated to pH 3.0 and treated as F_2)	
P_4, dissolve in H_2O and dilute to 10 mg. N/ml. Titrate to pH 9.0 (faint pink to phenolphthalein).	
Titrate to pH 4.0 after 1 to 2 min.	P_7
Centrifuge and wash with 0.002 N H_2SO_4	
Supernatant and washings, discard.	
Ppt., stir with water to thick paste and add 4 volumes per cent P_8 solution.	
Dialyze against 0.001 HCl at 20°C. until clear solution (3 to 4 days)	P_9
P_9, precipitate by addition saturated Na_2SO_4 solution.	
Filter. Ppt., dissolve with M/10 pH 5.0 citrate buffer and dilute so that solution contains about 5 mg. N/ml. Precipitate by addition of Na_2SO_4. This fractional precipitation is continued until no further increase in activity is shown by precipitate.	P_{10}

course of the several hundred precipitations required to reach the maximum activity. A large number of attempts were made to devise a rapid and efficient method of separation of the "gelatinase," without any success. No sharp separation could be found but a method was eventually worked out which although laborious allowed the preparation of an extremely active fraction with very little loss in activity. Most of the crystalline pepsin was removed by crystallization. The filtrate from the crystallization was precipitated by the addition of acid and the resulting precipitate dissolved with weak alkali and reprecipitated with acid. This process was repeated until the gelatin-liquefying power had been increased five to ten times. The material was then dissolved in water, brought to pH 9 and then acidified. The pepsin protein is denatured at pH 9.0 and precipitates on acidification, carrying with it the "gelatinase." A small amount of active pepsin is added to this precipitate and the mixture dialyzed against 1/100 M hydrochloric acid. Under these conditions the denatured pepsin protein is quite rapidly digested and passes out through the membrane leaving the "gelatinase." The solution obtained in this way is then fractionated by precipitation with sodium sulfate. About 30 per cent of the total gelatin-liquefying activity present in the original material may be obtained by this method. Details of the method of fractionation are shown in Table I.

TABLE II

Reactivation of "Gelatinase"

100 ml. P_3 solution (8 mg. N/ml.) titrate to pH 11.5 + NaOH. Titrate to pH 7.0 + HCl. Dilute with M/100 citrate buffer of different pH. Stand at 22°C. for 24 hrs.

pH	4.0		5.3		5.6		5.9		6.2	
Final concentration, *mg.* N/*ml.*	3	1	3	1	3	1	3	1	3	1
Percentage original activity recovered	<0.1	<0.1	2	6	2.5	10	5	15	5	12

The final fraction is a protein and loses its activity by heating or by the addition of strong alkali. Part of the activity can be recovered after alkali inactivation by titrating to pH 6. The results

TABLE III

Specific Activity of Various Pepsin Fractions. $[PU]_{gm\ N}$

Pepsin fraction……		Parke, Davis	Crystalline	P_3	P_6	P_7 (alkali inact.)	P_{18}	"Gelatinase"
Protein	Method							
Gelatin	2.5 per cent, pH 5.0 —viscosity $[PU]_{gm.\ N} \times 10^{-5}$	0.056	0.13	0.40	0.77	0.64	16	60
	5 " " pH 2.5 " " "	0.16	0.44	1.0	1.0	0.5	17	
	Formol $[PU]_{gm.\ N}$	0.4	1.1	0.6	1.0	0.4	1.7	
Casein	5 " " pH 2.5 —viscosity $[PU]_{gm.\ N} \times 10^{-6}$	2.8	12	8.5	6	0.1	3.5	
	+ " " "	0.3	1.5	2.0	1.2	0.01	0.5	
	Formol $[PU]_{gm.\ N}$	16	90	60	40	0.8	20	
	NPN "	100	440	290	200	6.6	120	
Milk	Rennet $[PU]_{gm.\ N} \times 10^{-7}$	5.3	28		10	0.22	7.40	
Egg albumin	5 " " pH 2.0 —viscosity $[PU]_{gm\ N} \times 10^{-5}$	0.5	1.4				0.3	
	+ " " "	0.001	0.01				0.002	
		0.002						
	Formol $[PU]_{gm.\ N}$	17	50				20	
		25	60					
	NPN "	44	120				30	
		30						
Edestin	5 " " pH 1.9 —viscosity $[PU]_{gm.\ N} \times 10^{-6}$	1.2	5.7				1.8	
	Formol $[PU]_{gm.\ N}$	25						
		30	80				35	
	NPN "	70	400					

of an experiment in which about 10 per cent of the activity was recovered are shown in Table II. The result is probably due to the reversal of denaturation of the protein.

Activity of Various Fractions

The activities of some of the fractions obtained in the course of the preparation with casein, egg albumin, edestin and gelatin were determined, as was also the effect on the rate of clotting of milk. Owing to the great difficulty in preparing the most active material the determinations were not carried out with this fraction but only with the fraction having about ¼ the maximum activity so far obtained. The results of these determinations are shown in Table III in which the activity per gram of nitrogen has been tabulated against the various fractions. The table shows that the activity of the crystalline pepsin is about five times that of the original material in every respect except the hydrolysis of gelatin. In this respect it is 2½ times as active as the original material. The amorphous precipitates obtained from the mother liquor of the crystallization are less active than the crystals

TABLE IV

Activity of Crude Pepsin and of "Gelatinase" as Per Cent of the Activity of Crystalline Pepsin

Protein	Method	Parke, Davis pepsin	"Gelatinase" P_{10}
Gelatin	2.5 per cent, pH 5.0 −viscosity	40	12,000
	5 " " pH 2.5 "	36	3,800
	Formol	36	150
Casein	5 " " pH 2.0 −viscosity	23	30
	+ "	20	35
	Formol	18	22
	NPN	23	27
Milk	Rennet	19	27
Egg albumin	5 " " pH 2.0 −viscosity	35	21
	+ "	20	20
	Formol	40	40
	NPN	30	25
Edestin	5 " " pH 1.9 −viscosity	21	32
	Formol	30	42
	NPN	18	

TABLE V

Activity of Pepsin Fractions by Different Methods Expressed as Per Cent of the Activity Measured by the Increase in NPN with Casein

Pepsin..........		Parke, Davis	Crystalline	"Gelatinase" P_{18}
Protein	Method			
Gelatin	2.5 per cent, pH 4.7 –viscosity	0.56×10^4	0.3×10^4	130×10^4
	5 " " pH 2.5	1.6×10^4	1.0×10^4	140×10^4
	Formol	0.4	0.25	1.4
Casein	5 " " pH 2.2 –viscosity	28×10^4	27×10^4	29×10^4
	+ "	3×10^4	3.3×10^4	4×10^4
	Formol	16	20	16
	NPN	[100]	[100]	[100]
Milk	Rennet	5.3×10^7	6.3×10^7	6×10^7
Egg albumin	5 " " pH 2.2 –viscosity	5×10^4	3.2×10^4	2.6×10^4
	+ "	0.02×10^4	0.02×10^4	0.017×10^4
	Formol	23	12	16
	NPN	36	27	25
Edestin	5 " " pH 1.9 –viscosity	12×10^4	13×10^4	15×10^4
	Formol	20	18	29
	NPN	70	90	

in their action on casein edestin and egg albumin solutions, but more active in their action on gelatin. As the fractionation continues this difference becomes more marked.

When the material is inactivated by alkali all but a few per cent of the activity as measured by the digestion of casein, edestin, or egg albumin is lost, while only about $\frac{1}{2}$ of the gelatin activity is lost. After dialysis and further precipitation the specific activity as measured by coagulation of milk or by the effect on casein, egg albumin, or edestin solutions decreases until it is about the same as that of the original crude material, while at the same time the gelatin-hydrolyzing power increases rapidly, especially when measured by the change in viscosity. These changes may be seen more clearly from Table IV, in which the results are expressed as *per cent* of the activity of the crystalline pepsin.

In Table V the results have been calculated as *per cent* of the activity as measured by the increase in non-protein nitrogen for casein. That is, the activity as determined by the increase in non-protein nitrogen for casein is taken as 100 and the activity as measured in other ways expressed as *per cent* of this value. The table shows that the most marked change in the relative activity as expressed as per cent of casein digestion again occurs in the case of gelatin. In this case the activity of the original preparation as measured by the change in viscosity is about 1×10^4 per cent of that as measured by casein digestion, while in the "gelatinase" fraction it is about 130×10^4 per cent.[1] This difference in the properties of the "gelatinase" and crystalline pepsin may be shown more clearly by plotting the increase in formol titration against the viscosity of the solution, as was done in the study of the changes in gelatin solutions caused by pepsin digestion (2). The result of an experiment done in this way is shown in Fig. 2. The figure shows that the "gelatinase" causes a relatively enormous decrease in the viscosity of the gelatin solution with a very slight increase in formol titration, while in the case of the original material or the crystalline pepsin there is a much larger increase in formol titration for the same decrease in viscosity. In the case of the "gelatinase" the viscosity is decreased nearly 50 per cent before any change in the formol titration can be detected by the method used here, which means that there is a very large change in the physical condition of the gelatin solution for a very small chemical change, or else that there are chemical changes in the gelatin which do not result in the liberation of free amino groups. It is probable that the chemical changes start at once and that they could be detected by the change in conductivity or some other more sensitive method. On the other hand, it follows from the structure of gelatin solutions as suggested by Kunitz and the writer (3) that a very slight amount of hydrolysis, especially if it were restricted to the insoluble fraction of gelatin, might cause extremely great changes in viscosity.

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[1] Since the activity units for the various methods have different dimensions, these percentage figures have no physical significance but are merely comparative. The activity as determined by any one method, however, is commensurable and may be compared directly, so that in this case the percentages are a measure of the relative activity by this method.

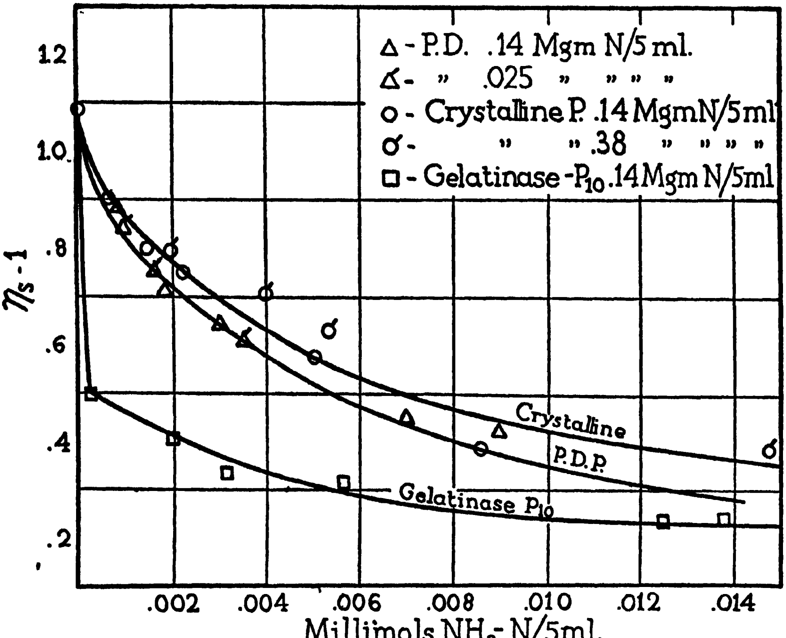

FIG. 2. Change in viscosity compared to the increase in amino nitrogen of Parke, Davis pepsin, crystalline pepsin and "gelatinase."

These experiments indicate that the activity of the "gelatinase" fraction on proteins other than gelatin is due to the presence of some pepsin, and that if this were removed the "gelatinase" would act only on gelatin. If this were the case it would be expected that repeated alkali-inactivation would destroy the activity on the other proteins. If the "gelatinase" is brought to pH 9.5 there is a considerable loss of activity with casein or edestin, but repetition of this procedure, causes only a slight additional loss. The result of an experiment of this kind is shown in Table VI. It shows that part of the casein- and edestin-digestive activity is more unstable than the gelatin-liquefying activity, but that some of the activity for casein and edestin has about the same stability as that for gelatin.

TABLE VI

Alkali Inactivation of P_{10}

	Per cent activity		
	Gelatin V.	Casein V.	Edestin V.
P_{10} solution, 0.10 *mg.* N/*ml*.	100	100	100
Titrate to pH 9.5 for 1 min. and then to pH 5.0	100	35	37
Titrate to pH 10.0 for 1 min. and then to pH 5.0	90	30	27

Changes in the Properties of Casein Solutions.—The addition of pepsin to acid casein solutions causes at first a very rapid decrease in viscosity, followed by an increase. Table V shows that the "gelatinase" fraction causes the viscosity to increase relatively more rapidly than does the crystalline pepsin or the Parke, Davis pepsin.

Experiments of a similar kind have been performed by Holter (4) who found that various pepsins differed in the way in which they affected the viscosity of casein as compared to their effect as measured by the titration of carboxyl groups. Holter plotted the increase in acid groups against the changes in viscosity and obtained characteristic curves for the various pepsin preparations. A series of experiments were carried out therefore in which the change in viscosity, the change in formol titration, and the increase in nitrogen not precipitated with trichloracetic acid (NPN) were determined. The results of these determinations are shown in Fig. 3, in which the specific viscosity of the solution has been plotted against the milli-equivalents of non-protein nitrogen per 5 ml. of solution. The figure shows that the "gelatinase" fraction causes a greater increase in viscosity for the same increase in non-protein nitrogen than does the crystalline pepsin. The original material gives results very close to those for the crystalline pepsin. The formol titration gave similar results, but the measurement is not as reliable. These curves are similar to those obtained by Holter for fractions separated by means of aluminum hydroxide, and indicate the possibility that the "gelatinase" fraction obtained in these experiments is the same as that

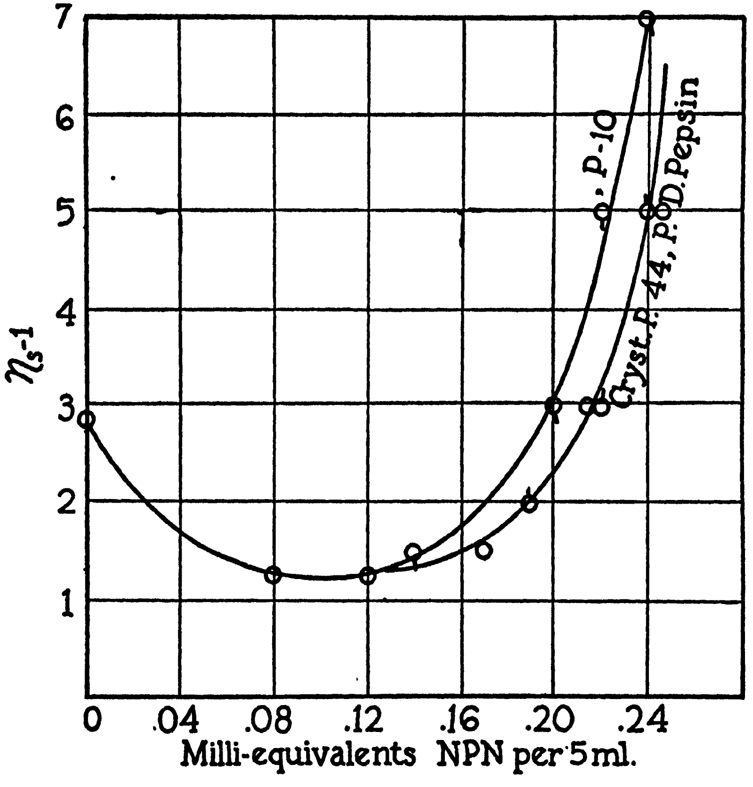

FIG. 3. Change in viscosity as compared to formation of non-protein nitrogen with crystalline pepsin, Parke, Davis pepsin and "gelatinase."

obtained by Holter by the use of aluminum hydroxide. Holter, however, states that a fraction may be obtained from crystalline pepsin, which shows the same peculiarity, by means of aluminum hydroxide. As was stated before, the "gelatinase" may also be prepared from crystalline pepsin after one or two crystallizations but not after four or five crystallizations.

These experiments were not carried out in greater detail. It will be noted that the viscosity of egg albumin also decreases and then increases while the viscosity of edestin solutions decreases only. No indication of an increase in the viscosity of edestin solutions could be found even with very high concentrations of pepsin.

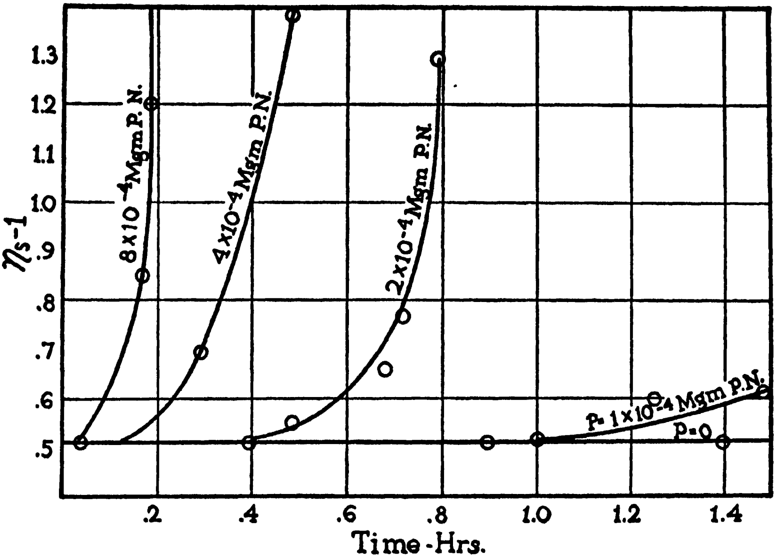

FIG. 4. Change in viscosity of milk with various concentrations of crystalline pepsin.

The activity of the various fractions as measured with egg albumin and edestin is about the same when expressed as per cent of the casein digesting activity (Table V).

Activity Units.—In the preceding paper (1) the activity was expressed as milli-equivalents of amino nitrogen liberated per minute under standard conditions. The unit of activity as measured by change of viscosity was based on this measurement of the increase in amino nitrogen. Since in the present work the activity as measured by the change of viscosity is found to vary independently of the activity as determined in other ways, it is necessary to have an independent unit for the activity as measured by viscosity changes. Since the percentage changes in viscosity of protein solutions of various concentrations are nearly independent of the protein concentration, it seems better to define the activity in terms of percentage change in the additional viscosity rather than absolute change. The activity units used in this paper are defined as follows:

Change in Viscosity:

The quantity of enzyme which, when added to 5 ml. protein solution, will cause a change of 1 per cent per minute at 35.5°C. in the additional viscosity.

a. Negative = $[\mathrm{PU}]^{\text{Protein V.}-} = \dfrac{100\,(T_0 - T_t)}{t_{\text{min.}}\,(T_0 - \mathrm{T}_{\mathrm{H_2O}})}$

$T_{\mathrm{H_2O}}$ = Time outflow of water.

T_0 = " " " solution at time O.

T_t = " " " " " " t.

t = Elapsed time in minutes since addition of enzyme.

b. Positive = $[\mathrm{PU}]^{\text{Protein V}+}$ = Per cent change in additional viscosity of protein solution per minute, at 35.5°C. when viscosity has returned to its original value.

Non-Protein N = NPN = $[\mathrm{PU}]^{\text{Protein NPN}}$ = Milliequivalents nitrogen not precipitated by 10 per cent trichloracetic acid per 5 ml. solution per minute at 35.5°C.

Formol = $[\mathrm{PU}]^{\text{Protein F}}$ = Milliequivalents amino nitrogen per 5 ml. solution liberated per minute at 35.5°C.

Rennet Action = $[\mathrm{PU}]^{\mathrm{R}} = \frac{100}{t_{\text{min.}}}$; Per cent change in additional viscosity of standard milk, calculated from the point at which the additional viscosity has doubled.

$T_{\text{min.}}$ = Elapsed time at which $\Delta\eta = 2\,(\eta_0 - 1)$

The negative viscosity measurements were made at short intervals with an Ostwald viscosimeter and the values determined in this way plotted against the elapsed time. A line is drawn through these points and the initial change in viscosity per minute determined from the slope of this line. The measurements are confined to the first 5 per cent change in viscosity, and in this range the change in viscosity per unit of time is constant except in the case of egg albumin. In all cases the determinations were made with very low concentrations of enzyme and the specific activity was independent of the quantity of enzyme used.

The positive viscosity rate was determined by plotting the viscosity against the elapsed time, and determining the slope of this curve at the point at which the viscosity had returned to its original value.

The increase in formol titration and non-protein nitrogen was determined by analyzing 5 ml. samples after 1, 2 and 3 minutes and the initial rate determined by interpolation from the curves.

The rennet activity was determined by plotting the change in viscosity against the elapsed time and determining by interpolation the time at which the increase in viscosity was equal to the original specific viscosity. 100 times the reciprocal of this time in minutes is taken as equal to the number of pepsin units as measured by rennet action.

EXPERIMENTAL METHODS

The experimental procedure and the protein solutions used in these experiments were in general the same as those described in the preceding paper (1).

The rennet action, however, was determined this time by following the changes in viscosity of a standard solution of commercial milk powder. This was prepared by dissolving 10 gm. of "Klim" milk powder in water and making up to 100 ml. 10 ml. of a molar solution of pH 5 acetate buffer is then added. 0.2 ml. of the pepsin preparation is added to 5 ml. of this standard milk and the change in viscosity at 35.5°C. determined.

The changes in the viscosity of isoelectric gelatin solutions are the most reliable and accurate. The changes in the viscosity of the solutions of edestin and casein are also accurate and reliable for comparative determinations but differ somewhat with various protein preparations. The determinations with egg albumin are the least satisfactory, in that all the methods show a relatively rapid change at the very beginning of the reaction which then becomes very much slower. Considerable variation was noted also with different egg albumin solutions, and the results with this protein may not be correct except as to their order of magnitude.

SUMMARY

A protein fraction has been isolated from crude pepsin preparations which is about 400 times as active as crystalline pepsin in the liquefaction of gelatin.

The activity as measured by the digestion of casein, edestin or egg albumin is less than that of crystalline pepsin.

It is more resistant to alkali than the crystalline pepsin.

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FURTHER STUDIES ON THE GLASS ELECTRODE

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In a series of papers from this Laboratory we have described a type of glass electrode which combines compactness with comparatively low resistance, its applicability to potentiometric titrations,[1] the use of the electrode for the determination of the *P*H of very small volumes of solution,[2] and also a search for a glass of suitable properties to serve as electrodes.[3] In this last investigation a glass was found which has a relatively low resistance, shows small potentials in the glass membranes, and can be used over a wide *P*H range to measure hydrogen-ion activities.[4] Direct comparisons between hydrogen gas electrodes and the glass electrodes were made in solutions which were given a series of *P*H values. The present paper describes an extension of those measurements, using a considerably improved apparatus. The precision and reproducibility of the electrodes have also been studied by measuring the *P*H of standard buffers and comparing the results with the usual form of hydrogen electrode.

Measurements have been made of the resistance, and the "asymmetry potential" of the electrodes and of the effect of heat and drying agents. Finally, the different theories of the operation of the glass electrode are considered in the light of the new experimental work.

The Construction of the Electrodes.—As explained in previous papers, the type of electrode used (G of Fig. 2) consists of a membrane of suitable glass of the order of 0.001 mm. thickness sealed over the end of a harder glass tube, of, say, 5 mm. diameter. Into this is placed 0.1 *N* hydrochloric acid and a silver-silver chloride electrode.

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[1] MacInnes and Dole, *Ind. and Eng. Chem.* (*Analytical Edition*), 1, 57 (1929).

[2] MacInnes and Dole, *J. Gen. Physiol.*, 12, 805 (1929).

[3] MacInnes and Dole, THIS JOURNAL, 52, 29 (1930).

[4] Glass made from this formula may be obtained from the Corning Glass Co., Corning, N. Y. (Their Number 015).

Since some difficulty has been reported in following the very brief directions already published, a few additional suggestions will be given below.

A small globule is melted on the end of a tube of Corning 015 glass with a gas-air blast lamp. This globule can then be blown into a bubble thin enough to show interference colors. Such a bubble will have thicker portions, indicated by their lack of color and relative rigidity, which may be used if desired. The proper temperature for this operation can only be found by experience. A tube of ordinary soft glass on which some of this glass membrane is to be mounted is then heated on one end to low redness, and is then placed in contact with the bubble of glass. It is well to cut this mounting tube with a sharp file or glass knife in order to get a flat even end. A large proportion of successful electrodes will be obtained if the mounting tube is brought into contact with the bubble with as little distortion or breakage of the latter as possible. That is to say, the mounting tube should be held as nearly as possible at right angles to, and its heated end in, the surface of the bubble. The bubble and mounting tube are separated only after the latter has had an opportunity to cool down somewhat. This separation necessarily breaks up the glass bubble. Considerable experience indicates that it is a waste of time to attempt to make more than one electrode from a glass bubble, since the approximately spherical shape of the latter is necessary for the support

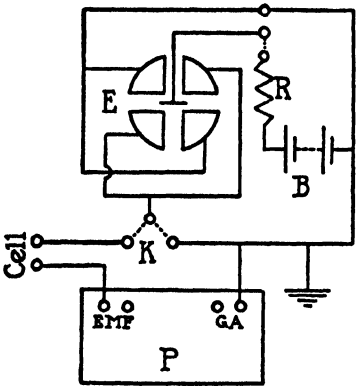

FIG. 1

of the membrane until it can be mounted. Loose pieces of membrane are, in general, too much disturbed by air currents to permit an effective seal onto a mounting tube.

The Electrical Measuring Apparatus.—A number of changes and improvements in the details of the apparatus for measuring the potentials have been made since the publication of the last paper. A diagram of the electrical connections is given in Fig. 1. We have used a Compton electrometer, E, together with a Type K Leeds and Northrup potentiometer, P.[5] Because of internal shunts in the potentiometer, the electrometer cannot be placed across the galvanometer terminals. An external circuit is, therefore, provided between the e. m. f. and galvanometer connections as is indicated in the diagram. The tapping key K of this external circuit impresses the net potential of the cell and potentiometer on two of the quadrants of the electrometer and on release automatically grounds them. To adjust the potentiometer, a galvanometer is connected to the usual binding posts. Under these conditions the potentiometer can also be used for ordinary e. m. f. measurements without disturbing the electrometer connections. The needle of the electrometer is charged from a battery B of from 45 to 90 volts. For protection this circuit is provided with a megohm resistance R. The potentiometer P is placed in a covered box lined with grounded copper screen. An arrangement is provided by which the final setting of the wire and the potential reading can be made without opening the box. The apparatus containing the glass electrodes is placed in a large box made of 6.4 mm. wire mesh, grounded. The Compton electrometer E is placed on a Julius suspension supported by crossed tape wound springs, as recommended by Johnsrud.[6] The suspension is surrounded by a grounded galvanized iron box to protect it from air currents and to afford additional screening. This box also holds the battery B. The electrometer has a sensitivity of from 12 to 23 millimeters per millivolt at 1 meter distance. The zero is steady and shows very little drift with time. All connections, including those to the galvanometer, are made with wire armored with woven copper wire, the latter being, of course, grounded. The insulation of this wire has been measured by Dr. S. E. Hill of this Institute and found to have a resistance between the wire and metal shielding of 10^{15} ohms per linear foot. This was also found to be the minimum resistance to ground of any part of the system from the cell to the electrometer. This careful screening and insulation make it possible to study electrodes with higher resistances than those considered in our previous papers. Effective insulation is desirable because an electrical leak which tends to shunt the cell containing the glass electrode will greatly affect the measurements by producing polarization. This is especially the case when dealing with high resistance electrodes. A reversing switch in the leads between the cell and the rest of the apparatus is sometimes convenient, but must have a very high resistance between opposite leads; otherwise it may be a source of trouble. The chief difficulties we have encountered with the apparatus were located in the imperfect insulation of a reversing switch and a tapping key.

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[5] Numerous papers have appeared on the use of vacuum tube circuits in connection with glass electrode measurements, the most recent being Stadie, O'Brien and Laug, *J. Biol. Chem.*, **41**, 243 (1931); Greville and Maclagan, *Trans. Faraday Soc.*, **27**, 210 (1931); and Hill, *Science*, **73**, 529 (1931). Our early experiments with such arrangements (mentioned in our first paper) indicated them to be inferior to the Compton electrometer in that currents which polarized the electrodes could arise from insufficient insulation and from other causes, and there was an annoying zero shift. These difficulties appear to have been largely surmounted by the development of improved vacuum tubes and of properly designed electrical circuits. However, none of these arrangements possesses the extreme simplicity of the electrometer, which in its present form is an adequately sensitive and thoroughly reliable instrument.

All measurements were made in a constant temperature room kept at 25 ±0.1°.

The Apparatus for Comparing the Glass and Hydrogen Electrodes.—The apparatus described below, for comparing the glass and hydrogen electrodes, is an improvement on the one outlined in the previous paper in that it is possible to obtain more accurate *P*H values with it and to know the pressure of the hydrogen used more accurately. It is also more convenient in use. The essential parts of the apparatus are shown in Fig. 2. The vessel A contains the solution into which are inserted the glass electrode G and the hydrogen electrode H. The *P*H of this solution may be changed by the addition of acid or alkali from the buret B. This buret is supplied from a special flask (not shown) with solution saturated with hydrogen gas of exceptional purity. Since no oxygen is admitted with the added solution, the adjustment of the hydrogen electrode to a new *P*H value is quite rapid. A stream of the purified hydrogen gas bubbles through the solution from the tube H_2, and passes out as shown through a hole in the support of the hydrogen electrode and the trap T. The *P*H measurements are made with reference to a 0.1 *N* potassium chloride calomel electrode E. To prevent contamination of the solution in this electrode with the saturated potassium chloride solution used as a bridge, the outlet tube of the half-cell dips into the tube D which can be filled with 0.1 *N* potassium chloride. To make a definite liquid junction, a saturated solution from the reservoir R can then be introduced under the tenth normal solution by manipulating the stopcock S. Only the outer edges of the barrel of this stopcock are lubricated. By another adjustment of S the tube D can be drained. A definite liquid junction between the solution in A and the saturated potassium chloride solution is made by an adaptation of the method used by Clark.[7] The tubing between the stopcocks S and O includes a piece of rubber

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[6] A. L. Johnsrud, *J. Optical Soc. Am.*, 10, 609 (1925).

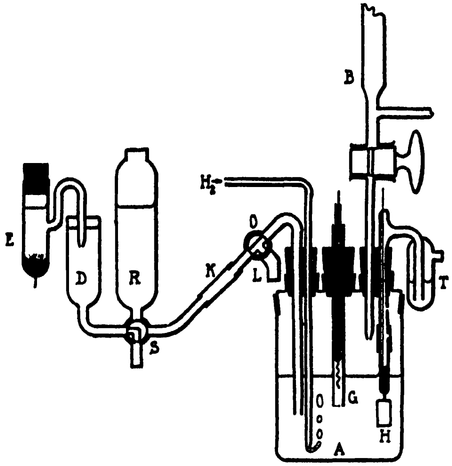

FIG. 2

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[7] W. Mansfield Clark, "The Determination of Hydrogen Ions," Williams & Wilkins Co., Baltimore, 1925, p. 296.

tubing K. The solution in this is first swept out through the tube L by adjusting S and O. S is then closed and a screw pinchcock screwed onto the rubber tube K. The stopcock O is then turned so that solution from A can be forced through L. The flow is brought about by raising the gas pressure in A by holding a finger over the outlet of T. The stopcock O is then turned so that the solution from A and the saturated potassium chloride are in contact and the pinchcock on K is opened. A fairly sharp liquid junction then forms just below the stopcock O.

The Experimental Results

As already stated, we desired to test the glass electrode against the hydrogen electrode at a series of P_H values under conditions which would yield a high degree of accuracy in the measurements. The P_H values were found from the e. m. f. between electrodes E and H of Fig. 2. These measurements give potentials, E_p of the cell

$$\text{Hg, HgCl, 0.1 } N \text{ KCl, KCl (satd.), soln. A, } H_2$$

in which soln. A is the fluid in vessel A. The P_H values will be *defined* by the usual equation

$$P_H = (E - 0.3376)/0.05915$$

although there is some uncertainty as to the accuracy of the constant used, and as to the need for correction of liquid junction potentials at high and low P_H values. The efficacy of the glass electrode for determining the hydrogen-ion activities was found by determining the potentials between electrodes G and H of the same figure. Such measurements yield potentials of the cell

$$H_2\text{, soln. A} \underset{a}{|} \text{ glass } \underset{b}{|} \text{ HCl (0.1 } N\text{), AgCl, Ag} \qquad \text{(A)}$$

In this cell there are five sources of potential: those at the hydrogen and silver chloride electrodes, the potentials at the surfaces *a* and *b* of the glass, and an "asymmetry potential" in the glass membrane. Now if the potentials at the glass surfaces *a* and *b* change with the P_H in the same way that the potential changes at a hydrogen gas electrode, the variations of e. m. f. due to altering of the acidity or alkalinity of solution A should cancel, and the measured potential should be that of the cell

$$H_2, HCl\ (0.1\ N), AgCl, Ag \qquad (B)$$

since the changes at the hydrogen electrode and at the glass surface *a* should be equal and in opposite directions.[8] Corrections must, however, be made for the asymmetry potential, and for the variation of the partial pressure of hydrogen from one atmosphere. On the other hand, any failure of the potential at the glass surface *a* to follow the changes of the hydrogen gas electrode quantitatively will result in a measurement of Cell A that differs from that of Cell B. Some quite typical results of measurements of cells of Type A are given in Table I, and indicate the nature and magnitude of the corrections which must

TABLE I

Difference of Potential between Hydrogen and Glass Electrodes Typical Data on E. M. F. of Cells

H_2, soln. A, glass, 0.1 *N* HCl, AgCl, Ag

Soln. A	PH of soln. A	Electrode	Observed e. m. f.	Correction for hydrogen pressure	Asymmetry potential	Corrected potential
0.1 HAc + NaOH	4.85	D13	0.3512	0.0004	+0.0004	0.3520
0.1 HAc + NaOH	6.54	D13	.3513	.0004	.0004	.3521
HAc + LiOH	6.90	D13	.3500	.0004	.0017	.3521
HAc + $Ba(OH)_2$	7.00	D13	.3505	.0004	.0012	.3521
HCl (0.1 *N*)	1.02	E13	.3497	.0004	.0020	.3521
HCl (0.1 *N*) + NaOH	4.25	E13	.3498	.0004	.0020	.3522
HAc (0.1) + $(C_2H_5)_4NOH$	4.87	D13	.3487	.0004	.0031	.3522
H_3PO_4	3.70	F	.3481	.0004	.0036	.3521

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[8] This can be shown as follows. Neglecting the asymmetry potential, the total potential *E* of Cell A is

$$E = 0 + RT/F\cdot\ln(H^+)_a - (E_{0g} + RT/F\cdot\ln(H^+)_a) + (E_{0g} + RT/F\cdot\ln(H^+)_{0.1})$$
$$-[E'_0 - RT/F\cdot\ln(Cl)_{0.1}] = -E'_0 + RT/F\cdot\ln(H^+)_{0.1}(Cl^-)_{0.1}$$

in which E_{0g} is the standard potential of hydrogen ion on the glass surface, E'_0 is the corresponding potential for the Ag-AgCl electrode and the terms in parentheses are the activities of the respective ions. Thus $(H^+)_a$ is the hydrogen-ion activity in solution A, and $(H^+)_{0.1}$ that in 0.1 *N* HCl. E_{0g} certainly changes from one sample of glass to another but is assumed constant for the two sides of the membrane.

be applied to the observed e. m. f. values. All the data in this table are taken in the *P*H range in which the glass electrode functions quantitatively. It will be seen that although solution A was widely varied in composition and *P*H, and several electrodes were used in the measurements, the corrected potential of the cell was 0.3521 ± 0.0001 volt. A direct measurement of Cell B gave us 0.3521 volt. Interpolation from the recent work of Randall and Young[9] yields exactly the same result.

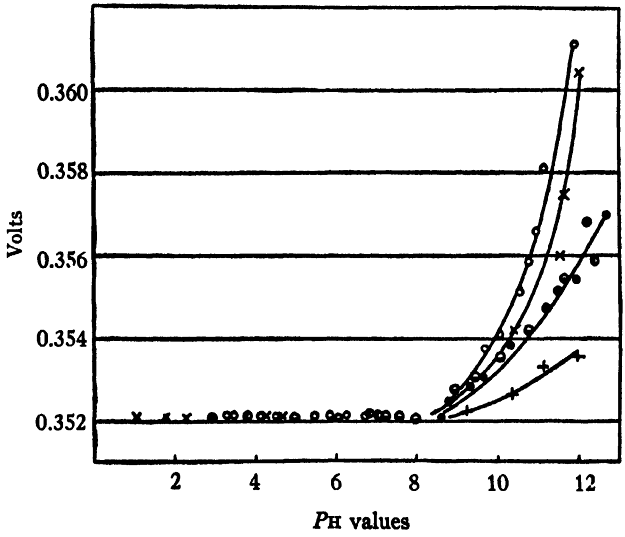

FIG. 3.—Difference between glass and hydrogen electrodes: ×, HCl + LiOH; o, HAc + NaOH; ⊖, HAc + LiOH; ◐, HAc + $Ba(OH)_2$; ●, HAc + $(C_2H_5)_4$ NOH; +, HAc + $Ca(OH)_2$.

More data of this type are given in Fig. 3, which represents the results of several series of measurements of cells of Type A in which the potential of the cell is plotted as ordinates against *P*H values as abscissas. In one of these series, solution A of the cell consisted of hydrochloric acid to which sodium hydroxide was added, from buret B of Fig. 2. Due to a lack of buffer action over part of the range this combination yielded points only in the higher and lower *P*H values. At the higher *P*H values there are large deviations. A very similar curve (not plotted) was obtained by the addition of lithium hydroxide at hydrochloric acid. To obtain more points at intermediate *P*H values, 0.1 *N* acetic acid was substituted for the hydrochloric acid, and to this were added solutions of a number of strong bases. Under these conditions the glass electrode follows the hydrogen gas electrode accurately up to *P*H 8. The results also show that up to this *P*H value the measured values of the difference between the glass and hydrogen electrodes were, when corrected for the asymmetry potential and the gas pressure, 0.3521 ± 0.0001 volt. This, it will be observed, is independent of the nature of the base and acid which made up the solution. MacInnes and Dole found agreement between the glass and hydrogen electrodes up to *P*H 9. This was, however, with glass of their own manufacture. At higher *P*H values deviations are observed, the values of which depend upon the nature of the base used. However, deviations when they occur are not reproducible, varying from electrode to electrode. Different deviations are found if the *P*H values are decreased from those obtained on increasing the alkalinity. Due to overlapping parts only of some of the curves are shown. The curve for the results obtained when tetraethylammonium hydroxide was used as base is also sufficiently representative of those found when tetramethylammonium hydroxide or guanidine hydroxide was substituted as base. About the only generalizations that can be made from the observations on the deviations are that the large complicated organic ions yield smaller deviations than do the ions of the alkali metals. Also divalent ions produce smaller deviations than do monovalent ions at the same *P*H values.

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[9] Randall and Young, THIS JOURNAL, 50, 989 (1928).

It next became of interest to find what is the lower limit of *P*H at which the glass electrode follows the hydrogen gas electrode. In these experiments a solution of about 0.01 *N* of the acid used was placed in vessel A of Fig. 2, and measurements of *P*H and the e. m. f. between the glass and hydrogen electrodes were then made. A strong solution of the acid was next added in successive portions from buret B, thus lowering the *P*H. The differences in e. m. f. between the hydrogen and glass electrodes are plotted in Fig. 4. At low *P*H values there is, for all the acids tested, a deviation in the opposite direction to that observed for high *P*H values. Furthermore, the deviation varies with the nature of the acid used, though as in the case of bases the variations are not very reproducible. The deviations produced by sulfuric acid are greater than those found with the monobasic acids tested, and the effect of phosphoric acid is still greater. We have not been able to find any other di- and tribasic acids, which are otherwise suitable, by which we could make additional tests of this apparent effect of valence type on the deviations observed. Suitable acids would have to be strong and have no adverse effect on the hydrogen electrode. There is, as far as we can determine, no evidence in previous publications that other workers have observed a lower limit of *P*H at which the glass electrode functions. Electrodes made of the glass used in these experiments are evidently not suitable for studying "superacid" solutions.

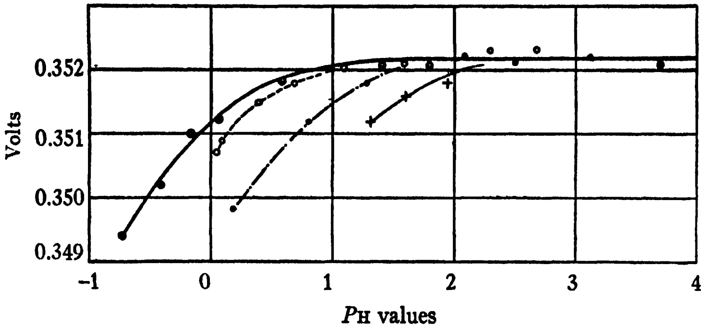

FIG. 4.—⊖, HCl; o, $HClO_4$; ●, H_2SO_4; +, H_3PO_4

Such experiments as we have just described have been designed to bring out and emphasize deviations when they occur. It should, however, be observed that these experiments indicate that the glass electrode functions quantitatively in the measurements of hydrogen-ion activity between *P*H values 2 and 8 within one or two tenths of a millivolt, *i.e.*, about 0.002 to 0.003 *P*H unit.

In order, however, to attain this order of accuracy, particular attention must be paid to the measurement of the asymmetry potential. Such measurements have been made by setting up the cell

Ag, AgCl, 0.1 *N* HCl | glass | 0.1 *N* HCl, AgCl, Ag (C)

one of the acid solutions being outside and the other inside the electrode tube. We have repeatedly found that the potential of a silver-

silver chloride electrode is constant only if the acid immediately on its surface is not disturbed. The measurements of Cell C were therefore made in a U-tube one arm of which held the glass electrode ensemble and the other a reference silver chloride electrode surrounded by a narrow tube which had a small opening at the bottom to allow for the passage of current.

The "Resistances" of Electrodes.—Although the opposition of the electrodes to the flow of electric current has been regarded up to the present time as ohmic resistance and has been reported in megohms, measurements by both direct and alternating current show clearly that at least the greater part of this apparent resistance is due to counter e. m. f., presumably due to polarization. Some typical measurements by both methods of a series of electrodes are given in Table II. It will be observed that the apparent resistances found by measurements using an alternating current of frequency 1200 are very much lower and roughly proportional to those obtained by d. c. determinations.

TABLE II

Comparison of A. C. (1200 Cycles) and D. C. Resistance Measurements on Glass Electrodes

No. of electrode	Apparent resistances	
	A. C. megohms	D. C. megohms
1	1.28	54.5
2	1.32	51.0
3	3.96	129.0
4	1.69	52.5
5	2.84	102.0

This striking difference between the results of a. c. and d. c. measurements has long been familiar to biologists from their studies of the electrical properties of surfaces of cells.

The d. c. measurements were made using an electrometer, as described in a previous paper.[9] The a. c. determinations were kindly carried out by Dr. Theodore Shedlovsky of this Laboratory, using a Wheatstone bridge described by him in a recent paper.[10] The electrodes were shunted with a 10,000 ohm resistance to bring the measurements in the range of the bridge and the a. c. resistances computed from the bridge readings by the equation for parallel circuits. The a. c. resistances are functions of the frequency as is shown in Table III for a typical electrode.

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[10] Theodore Shedlovsky, THIS JOURNAL, 52, 1793 (1930).

It will be seen that the apparent resistance decreases at first rapidly and then more slowly with increase of frequency. This change is due to the fact that the arm of the bridge, the impedance of which balances the impedance of the electrode and shunting resistance, does not imitate the latter sufficiently closely. The glass electrode has an effective capacity due to polarization in series with its ohmic resistance and, in addition, a geometric capacity in parallel due to the conducting electrolyte on each side of the glass membrane. Under these circumstances the results of resistance measurements will be a function of the frequency. However, the a. c. measurement is nearer than the d. c. determination to the true ohmic resistance.

TABLE III

Apparent Resistance of a Typical Electrode as Affected by Frequency of Measuring Current

Frequency	0	1020	1470	2050	3380
Apparent resistance, megohms	81	2.55	2.05	1.86	1.27

Although the apparent d. c. resistance must be nearly all due to a counter e. m. f. of polarization, it is an interesting fact that such a resistance is found to be nearly constant when measured using different applied potentials. In other words, Ohm's law is approximately followed.[11] The figures given in Table IV for one electrode are typical.

In view of these observations it does not appear desirable to change the current practice of reporting the apparent d. c. resistances of electrodes in megohms.

[11] Joffé [*Ann. Physik*, 72, 463 (1923)] has shown that for insulators the correct form of Ohm's law is $i = (E - P)/R$ in which i is the current, E the applied electromotive force, P the counter e. m. f. of polarization and R the true resistance. P is, however, a function of E. If we can assume, as a first approximation, that $P = kE$, where k is a constant, then $i = E(1 - k/R$, *i. e.*, Ohm's law will apparently hold. Sinjelnikoff and Walther [*Z. Physik*, 40, 786 (1927)] show that for mica the relation between P and E is approximately linear for the lower values of E.

It has been our experience that it is desirable to work with electrodes with as low apparent resistances as possible in spite of the greater fragility of thin membranes. If thick electrodes are used, difficulties arise in connection with insulation and electrical screening. Obviously, accuracy can only be attained if the resistance of the path of the current through the glass membrane is small compared with all other possible paths of the current. With thick electrodes leak paths may reach the order of magnitude of the path through the active film of glass. It is a matter of observation that measurements with thick electrodes are more influenced than with thin electrodes by electrical leaks. If the cell measured is shunted by a leak resistance which is high compared with the resistance of the glass electrode, the current through the leak will be nearly constant for electrodes of varying resistances. Under these circumstances the polarization of the electrodes will increase as the resistance of the electrodes increases.

TABLE IV

Apparent Resistance as Affected by the Applied E. M. F.

Applied e. m. f., volts	Current $\times 10^9$	Apparent resistance, megohms
0.126	1.02	123
.252	2.17	121
.377	3.12	121
.629	5.15	122
.879	7.26	121

One possible source of trouble, particularly with thick electrodes, may be the supporting tube. In our work this has always been of harder glass than that forming the membrane and of a composition such that its potential does not respond accurately to change of hydrogen-ion activity. It is therefore desirable to keep the flow of current through the supporting tube as small as possible. This can be accomplished by using relatively thick tubing of low conductance and by employing thin membranes. The resistance of ordinary soft lime glass is about five hundred times that of Corning 015 glass. This has been shown by making several electrodes with very thin membranes (about 0.001 mm.) of the soft lime glass. Their apparent d. c. resistance was about 2500 megohms. An equivalent electrode of 015 glass has an apparent resistance of about 5 megohms. Considering that the wall of the tube is about one millimeter thick, it does not appear that any appreciable current can pass through it. Additional evidence that the supporting tube is not a source of leakage or other trouble was shown as follows. Using the technique described in the next paragraph, the *P*H of a solution was determined with different immersions of the mounting tube of a glass electrode. The potential observed did not change as much as 0.1 millivolt from an immersion of one millimeter to one of eleven centimeters.

Measurements with Buffer Solutions.—It has seemed worth while to test the electrodes with standard buffers to determine their reproducibility under the conditions in which they will normally be used. The measurements were made with the aid of the electrode vessel shown in Fig. 5. The solution under observation was placed in the tube A, into which the glass electrode E could be plunged by means of a holder operated by a rack and pinion (not shown). A can be drained through tube C, and a liquid junction can be formed in tube D, with the aid of the rubber tube K. The rest of the apparatus is identical with the left-hand side of the apparatus shown in Fig. 2. Two of the buffer solutions tested were 0.05 *M* potassium acid phthalate, and a sodium veronal-hydrochloric acid mixture.

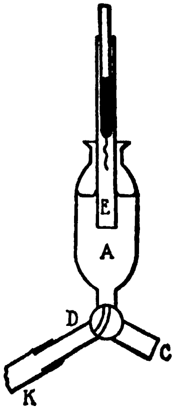

FIG. 5

Some results with the phthalate buffer, using six different electrodes, are given in Table V. These readings were made against a saturated calomel electrode,[12] using the formula $E = -0.1063 + 0.05915\ P_H$. As explained in the last paper in this series the constant used is the difference between Clark's "arbitrary value" of the standard potential of the saturated calomel electrode 0.2458 and the potential 0.3521 of Cell B (page 333). A similar series made with a 0.1 *N* potassium chloride calomel cell as reference, using −0.0145 (*i.e.*, 0.3376 − 0.3521) as the corresponding constant gave an average value of 3.982 for the *P*H of the phthalate solution. Direct measurement with the usual Clark hydrogen electrode apparatus yielded 3.983 for the *P*H value on

TABLE V

*P*H *Values of 0.05 M Potassium Acid Phthalate Buffer at 25°*

Electrode	Measured e. m. f	Asymmetry potential	*P*H
R23	0.1278	+0.0013	3.980
R13	.1276	.0015	3.980
R14	.1278	.0014	3.981
R20	.1283	.0008	3.980
R22	.1284	.0008	3.981
R 8	.1309	− .0017	3.981

TABLE VI

*P*H *Values of Sodium Veronal Buffer at 25°*

Electrode	Measured e. m. f.	Asymmetry potential	*P*H
A5	0.4594	−0.0006	8.000
H10	.4616	− .0029	7.998
H11	.4625	− .0041	7.993
H9	.4612	− .0028	7.993
T10	.4620	− .0032	8.000
M4	.4620	− .0035	7.995
T17	.4637	− .0051	7.996
C1	.4677	− .0089	8.000
			Average 7.997

A determination with the hydrogen electrode gave *P*H = 7.998.

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[12] The saturated calomel electrode appears to be as reproducible as the 0.1 *N* electrode provided that it can be kept for considerable periods at the temperature at which the measurements are made. Otherwise it shows hysteresis effects that may reach several millivolts.

the same basis. Clark and Lubs' original measurements[13] yield P_{H} = 3.98 at 20° if computed in the same way as for the above measurements.

The sodium veronal-hydrochloric acid mixture was prepared according to the directions of Michaelis[14] to give a P_{H} of 8. The results of the measurements with the glass electrode are given in Table VI.

These examples from a number which could be given indicate that if adequate precautions are taken, especially with the asymmetry potential measurements, the glass electrode will give very accurate determinations of hydrogen-ion activity within the range in which it is applicable.

Theory of the Glass Electrode

Theories concerning the operation of the glass electrode have been proposed by several authors. The theory of Haber and Klemensiewicz[15] amounts fundamentally to the assumption that a Donnan equilibrium exists across the glass membrane with the proviso that H^+ and OH^- ions can penetrate the glass and other ions cannot. As is well known, a membrane at which a Donnan equilibrium exists has a potential which changes reversibly with changes of the activities of the ions that are able to pass through it. Haber and Klemensiewicz state their assumptions in a somewhat different way. They consider a cell of the form

$$H_2,\ \begin{matrix}\text{acid}\\\text{solution}\end{matrix}\ \Bigg|\ (H^+)(OH^-) = K_w\ \Bigg|\ \begin{matrix}\text{alkali}\\\text{solution,}\end{matrix}\ H_2$$

In this cell two hydrogen electrodes are in two solutions which are separated by another phase, which has the property that the ion product (H^+) (OH^-) is equal to that of water. Thermodynamically this cell should have zero potential, which would also be true if the intermediate phase were a Donnan membrane in the usual sense. . We have shown that the operation of the glass electrode is intimately related to the water in the glass by means of some simple experiments. These experiments have shown that any process tending to dry out the electrode causes large increases in apparent resistance. For instance, a typical electrode had an apparent (d. c.) resistance of 32 megohms. After heating at 50° in vacuum with phosphorus pentoxide the resistance increased 40,000 megohms. The drying process can proceed even at room temperature, as is shown by the following observations. Six electrodes were placed in a vacuum desiccator over phosphorus pentoxide for ten days at 25°. As a control four electrodes were kept over water, but otherwise under the same conditions. The resistance measurements are given in Table VII.

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[13] Clark and Lubs, *J. Biol. Chem.*, **25**, 479 (1916).

[14] Michaelis, *ibid.*, **87**, 33 (1930).

[15] Haber and Klemensiewicz, *Z. physik. Chem.*, **67**, 385 (1909).

There is, it will be noted, a very much greater increase of resistance when the electrodes are kept in a dry atmosphere than when they are held in water vapor. The increase of resistance of the electrodes kept over water may be due to a slow crystallization of the glass. A still more marked increase of resistance due to drying took place at 39°. After immersion in water there was a slow return to approximately the original resistance.

TABLE VII

Changes in Apparent Resistance of Electrodes with and without Drying at 25°

Electrode	With drying Resistance, megohms		Electrode	Over water Resistance, megohms	
	At beginning	After 10 days		At beginning	After 10 days
1	28	113	1	22	30
2	28	60	2	28	38
3	36	118	3	42	56
4	27	99	4	41	51
5	24	93			
6	41	110			
Average increase with drying, 230%			Average increase without drying, 34%		

At room temperatures the movement of water molecules in and out of the glass must be very slow. This is shown by the fact that a week of drying at 25° did not produce more than a small fraction of the change of the apparent resistance found by drying at 50°. At the lower temperature the drying may have been confined to the outer layers only of the glass membrane.

That water passes very slowly through the glass was also shown by placing dehydrated copper sulfate in contact with a very thin membrane of a glass electrode, sealing off the mounting tube, and placing the outer surface of the membrane in water. Although a small amount of water would have changed the color of the copper sulfate from white to blue, there was no perceptible change of color in twenty days. More sensitive tests for the passage of water through thin glass membranes will be carried out in the near future.

That one of more of the ions of water can penetrate the glass under the influence of an electric potential is shown by the work of Quittner,[16] who found that the amount of current carried from an acidified salt solution by hydrogen ions increased rapidly with the percentage of acid present, being 100% at an acid concentration of 5×10^{-4}. Similar results were obtained in this Laboratory by L. L. Burgess, and will be presented later. He found that with very thin electrodes acid formed in accordance with Faraday's law at the glass surface. Either of these results could arise from the movement through the glass-liquid boundary of hydrion in the direction of the current or of hydroxyl ions moving in the reverse direction, although Quittner's observations make the penetration by hydroxyl ions appear quite improbable. Furthermore, we have just presented evidence that water may move in and out of the glass. It is thus possible for the theory advanced by Haber and Klemensiewicz to be correct. However, it involves the qualification that the glass must be in equilibrium with the water in the solutions with which it is in contact.

It seems to us that equilibrium of water throughout the glass is improbable because of the slowness with which water enters and leaves it. A simpler assumption, and one which accounts for all the phenomena within the *P*H range in which the glass electrode functions quantitatively, is that the hydrogen ion, or proton, can pass the electrolyte-glass boundary at a lower potential than any other positive ion and that negative ions do not pass the boundary at all. This does not involve any assumptions as to the conduction in the glass. It is quite probable that other ions than hydrogen move in the body of the glass.

Hughes[17] has proposed, as an explanation of the behavior of the glass electrode, the theory that the sodium silicate and excess silicic acid in the glass act as a buffer, preventing penetration of ions from the solution from changing the hydrogen-ion concentration of the glass. The failure of the buffer process, according to this author, begins at *P*H 9, which is about that of the first dissociation constant of silicic acid. If it were possible to make such an assumption, it would be helpful but not sufficient. A junction of the sort.

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[16] Quittner, *Ann. Physik*, [IV] 85, 745 (1928).

[17] Hughes, *J. Chem. Soc.*, 491 (1928).

soln. A | buffer

will give a potential following the hydrogen-ion activity only if the hydrogen ion is the only ion that can pass the boundary. Further, if, as is assumed by Hughes, the glass is a buffer consisting of a mixture of an acid with a dissociation constant of 10^{-9} and its neutral salt, it has the unusual property of maintaining its *P*H in contact with very strong acid, but of failing to do so in contact with solutions whose *P*H values are in the region of the hypothetical *P*H of the glass at its greatest "buffer value."[18] Furthermore, without additional assumptions it does not seem possible to deduce the hydrogen-ion concentration of glass from ionization constants applicable to aqueous solutions.

To account for the deviations at high and low *P*H values, the assumption of ionic interchanges in the surface appears to be sufficient. If the hydrogen-ion concentration of the solution in contact with the glass surface becomes very low, a high potential will be present between the hydrogen ion in the glass and that in the solution. Any reaction that will reduce this potential will tend to occur. A possible reaction from, for instance, a sodium hydroxide solution, will be

$$Na^+ + (H) = (Na) + H^+$$

the terms in parentheses representing the material present in the glass. It is also possible that the reverse of this reaction occurs at very high hydrogen-ion activities, and accounts for the deviations at low *P*H values. In this connection much light can be thrown on the observations of the behavior of glass at room temperature by a consideration of results of experiments by other workers which have been carried out at 200° and above. At these more elevated temperatures the glass is probably largely dehydrated and some other ion (usually sodium) takes the part played by hydrogen in the colder glass. In an important paper G. Schulze[19] showed that the reaction

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[18] Van Slyke, *J. Biol. Chem.*, **52**, 525 (1922).

$$Ag^+ + (Na) \rightleftharpoons (Ag) + Na^+$$

takes place spontaneously and quantitatively across the boundary between glass and molten silver nitrate. In this equation Ag^+ and Na^+ represent ions in the melt and (Na) and (Ag) the corresponding constituents in the glass. This demonstrates the reality of ionic interchanges at higher temperatures and makes them appear probable at lower ones. Furthermore Kraus and Darby[20] find that monovalent ions enter glass much more readily than do divalent ions. This observation has also been made by Quittner. It will be seen that in Fig. 3 the deviations produced by sodium and lithium are much greater than those found with the divalent barium and calcium ions, which apparently do not exchange so readily, possibly because one of the latter would have to occupy the position previously taken by two hydrogen ions in the glass. The potentials at the surfaces of glass electrodes have been studied from this point of view by Horovitz and associates.[21] This investigator has recently given a formula for the potentials resulting from such exchanges.[22] His treatment, which is very briefly outlined in the reference given, includes correction for the diffusion potential which would arise from the change of composition of the glass resulting from the ionic interchange. Although his formula predicts deviations of the general form shown in Fig. 3, the computed values tend to increase more rapidly than the observed values with the *P*H if the necessary empirical constant is obtained from the lower *P*H measurements. The equation does not predict deviations in strongly acid solutions.

As a working hypothesis, then, we consider that the known facts about the glass electrode may be explained by the following assumptions. The glass contains a small amount of water which yields hydrogen ions. These can move in and out of the surface of the glass more readily than any other ions. The glass thus acts as a reservoir for hydrogen ion of constant activity. There is no need for the activity of this hydrogen ion to be the same in different samples of glass. If, however, the potential due to the difference of chemical potential of the hydrogen ion at a glass surface and that in the solution becomes too high, ionic exchanges will occur which will tend to lower this potential. When such exchanges take place the glass surface ceases to function quantitatively as a hydrogen electrode. Further experiments designed to test this hypothesis are in progress. A study is also being made of the operation of the glass electrode in solutions which are very slightly buffered.

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[19] G. Schulze, *Ann. Physik*, [IV] 40, 335 (1913).

[20] Kraus and Darby, This Journal, 44, 2783 (1922).

[21] Horovitz, *Z. Physik*, 15, 368 (1923); *Sitzb. Akad. Wiss. Wien*, Abt. IIa, 134, 335 (1925); Horovitz and Zimmermann, *ibid.*, 134, 355 (1925).

[22] Horovitz, *Nature*, 127, 440 (1931).

SUMMARY

Comparisons of glass and hydrogen electrodes have been made through wide *P*H ranges under conditions designed to yield high accuracy in the measurements.

From *P*H 2 to 8 the two electrodes agree within less than ±0.0002 volt, if corrections are made for "asymmetry potential." Above *P*H 8 deviations occur which depend upon the nature of the positive ions present. Also at very low *P*H values deviations of an opposite kind are observed. These apparently depend upon the nature of the negative ions present in solution.

Resistance measurements made by a. c. methods on glass electrodes indicate that most of the apparent resistance obtained by d. c. measurements must be due to polarization.

Large increases of apparent resistance of the electrodes result from drying processes even at room temperature, indicating the importance of water, or its ions, in conduction through the glass membranes.

Theories designed to explain the operation of glass electrodes are considered in the light of these new experimental results.

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PYOCYANINE, AN ACCESSORY RESPIRATORY ENZYME

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In a previous study (1) it has been shown that pyocyanine in mixture with its leuco compound is a reversible oxidation-reduction system that at pH > 6 behaves like any one of the known reversible dyestuffs of quinoid structure. How this property may manifest itself under biological conditions is shown in the following experiment:

A culture of *Bacillus pyocyaneus* in which the blue pigment has been formed becomes colorless on the exclusion of air. On agitation with air the blue color is restored. The bacteria reduce the pigment to the colorless leuco base, which then by the oxygen of the air is reoxidized to the colored dye. Pyocyanine acts here as an autooxidizable acceptor for labile hydrogen of the microorganisms. This experiment shows that pyocyanine may play a rôle in dehydrogenative oxidation of bacteria in that it undergoes a cycle from the oxidized to the reduced and back to the oxidized form. As potentiometric studies (1) have shown that this cycle is also reversible thermodynamically, *i.e.*, in the course of this cycle the entropy of the system, pyocyanine-leucopyocyanine, remains constant, all the theoretical requirements are given which would allow pyocyanine to serve as an ideal catalyst for bacterial oxidation processes. But before pyocyanine can be so considered, proof is required that this pigment actually accelerates the oxygen consumption, *i.e.*, the respiration of bacteria. This proof is submitted in what follows:

Outline of the Experiment

The Warburg manometric method was applied to measure the respiration of *B. pyocyaneus* with and without the addition of pure recrystallized pyocyanine, using bacilli that had been cultivated under conditions impeding the formation of pigment and that had been washed free from all traces of nutritive media and suspended in a phosphate buffer.

Procedure.—The following strains of the American Type Culture Collection were used: (1) *Pseudomonas aeruginosa* No. 97, Army Medical School, Washington, D. C.; and (2) No. 256 E. O. Jordan, University of Chicago (Catalogue of Cultures, 1928). The two strains behaved in every respect identically. Cultures free from pigment, or producing only traces of the green fluorescent pigment were obtained at 30°C. in Blake bottles on lemco agar (0.3 per cent lemco, 0.5 per cent NaCl, 1 per cent Witte peptone, 2 per cent agar, pH 7.6). Cultures 15 hours to 5 days old were used.

The bacteria were swept off the medium and washed three times in the centrifuge with distilled water, and finally suspended in phosphate buffer (Sørensen). The suspension was made thoroughly homogeneous by filtration through paper and by shaking in a machine. A count of the bacterial suspension was not made as all measurements were comparative and hence the absolute quantity of bacteria was irrelevant. Therefore not the measured absolute values of respiration, but only the relative increases, are comparable for different experiments. 1 to 2 cc. of the bacterial suspension were placed in the main compartment of Warburg's microrespiration vessels. The small inner vessel was filled with 0.2 cc. of 6 per cent NaOH. Pyocyanine was purified by recrystallization out of chloroform and stocked in N/20 HCl. Before each experiment the necessary amount of stock solution was adjusted to the proper pH (around 7.0) by the addition of secondary sodium phosphate. This dye solution was then added to the bacterial suspension in the proportion of about 1 to 10. The final molar concentration of pyocyanine was 1/5,000 M in all experiments. All the work was carried out under aseptic conditions. Aerobic and anaerobic test cultures produced at the end of the experiments showed, firstly, that the bacilli were still living and capable of reproducing, and, secondly, that no contamination with foreign microorganisms had occurred.

RESULTS

Effect of Pyocyanine on the Respiration of B. pyocyaneus.—All experiments showed unequivocally that the bacilli, suspended in an indifferent phosphate buffer, consume a small but definitely measurable amount of oxygen. Addition of pyocyanine increases this amount up to many hundred per cent as can be seen in Table I.

Fig. 1 represents the results of a typical experiment. Control tests prove that under the conditions pyocyanine itself does not consume any oxygen. Only at strongly alkaline reaction, beyond pH about 9.4, does pyocyanine oxidize spontaneously and irreversibly.

Effect of Temperature.—The figures given above were found at temperatures ranging from 18–24°C. Elevation of temperature up to 37°C. increases the basic oxygen consumption of the bacteria alone.

The increase brought about by pyocyanine over this already increased respiration, is not so large as at the lower temperatures, but is still quite important: 29 per cent to 127 per cent.

Effect of pH.—Fig. 2 shows that the increase of respiration induced by pyocyanine is about constant between the tested limits of pH, 5.3 and 7.7. The increase of the pyocyanine respiration observed between pH 5.3 and 7.2 parallels approximately the increase in the basic respiration of the bacteria alone.

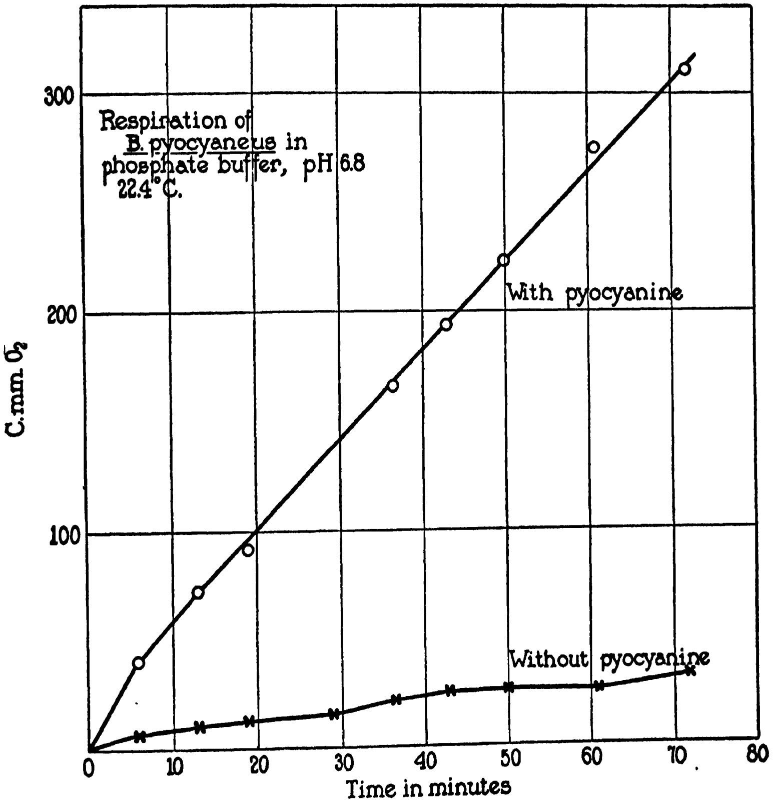

FIG. 1

The Oxygen Consumption of Killed Bacilli.—Does the excess consumption of oxygen brought about by pyocyanine really signify an increase in respiration of the living bacteria or does the extra oxygen serve only for the oxidation of some bacterial product, in the sense of the accessory respiration (*Nebenatmung*) of Batelli and Stern (2)? Bacteria killed by 1 hour exposure to 56°C., or by the addition of tolu-

TABLE I

Oxygen Consumption of Washed B. pyocyaneus Suspended in Phosphate Buffer

No.	pH	Temp.	Oxygen consumed		Increased respiration
			With pyocyanine	Without pyocyanine	
		°C.	*c.mm.*	*c.mm.*	*per cent*
1	7.4	21.4	− 4	− 51	1,275
2	7.4	21.4	− 4	− 41	1,025
3	6.8	22.3	− 32	−310	970
4	6.8	22.2	−527	−290	550
5	6.9	20.9	− 4	− 96	2,400

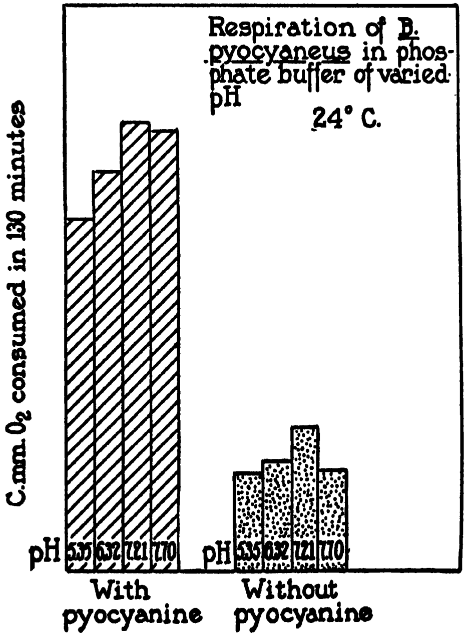

FIG. 2

ene did not show any oxygen consumption at all, either with or without pyocyanine (see Fig. 3). Therefore it may be concluded that pyocyanine affects actually the respiration (*Hauptatmung*) of the living bacteria.

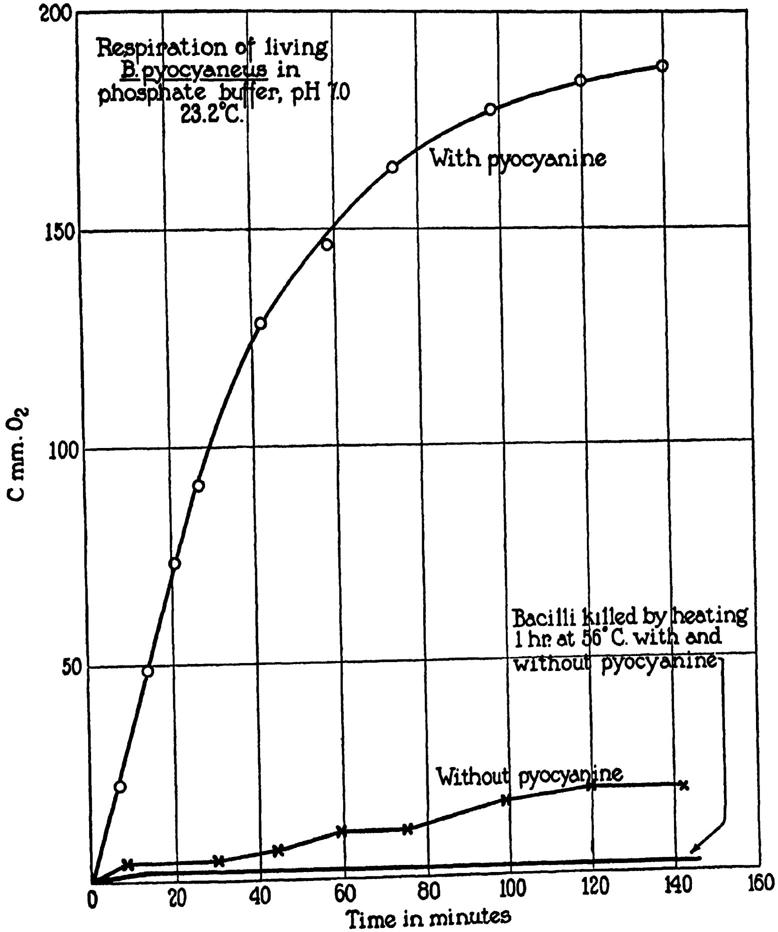

FIG. 3

Effect of Pyocyanine on the Respiratory Quotient.—Is the increase of respiration induced by pyocyanine purely quantitative or does pyocyanine affect also the process of oxidation qualitatively? With a view to elucidating this question the respiratory quotient was determined by the method of Warburg and Yabusoe (3). As shown in Table II, it was found that without pigment and even when appreciable amounts of oxygen are consumed, the formation of carbon dioxide is very small; but it is enhanced by the addition of pyocyanine to such extent that the respiratory quotient increases many times and approaches 1.0. It appears thus that the oxidations brought about by the bacteria without the pigment are to a great extent incomplete, but that they can be completed with its assistance.

Of What Substances Is the Oxidation Catalyzed by Pyocyanine?—Since in all the experiments described above the bacteria were freed through washing of all traces of the nutritive media, only such substances as are part of the microorganisms can come under consideration. This conclusion is sustained by the analysis of the curves showing the oxygen consumption as a function of time. As Fig. 3 shows, the curve for oxygen consumption under the influence of pyocyanine progresses during the 1st hour in a straight line, *i.e.*, the consumption is directly proportional to time, but thereafter it falls off more and more, the curve tending to become parallel to the abscissa. But, in the presence of an assimilable substance, such as glucose, asparagin, or pyruvic acid, the curve of oxygen consumption continues to progress in a straight line (Fig. 4). It follows that the flattening of the curve just mentioned cannot be caused by a diminution of the partial oxygen pressure, but is the consequence of an exhaustion of oxidizable substances. Furthermore it cannot be that the oxidation of these added substances is catalyzed by pyocyanine because if they are added without pigment to the washed bacteria, the respiration also increases many times. Under such circumstances the subsequent addition of

TABLE II

Respiratory Quotient of Washed B. pyocyaneus Suspended in Phosphate Buffer

With pyocyanine	Without pyocyanine	Temp.	pH
		°C.	
0.77	0.17	22.2	6.8
0.9	0.1	20.9	5.8

pyocyanine does not appreciably increase the respiration which has already been enhanced. This falls in with the findings on the influence of higher temperature reported in a previous paragraph. It may

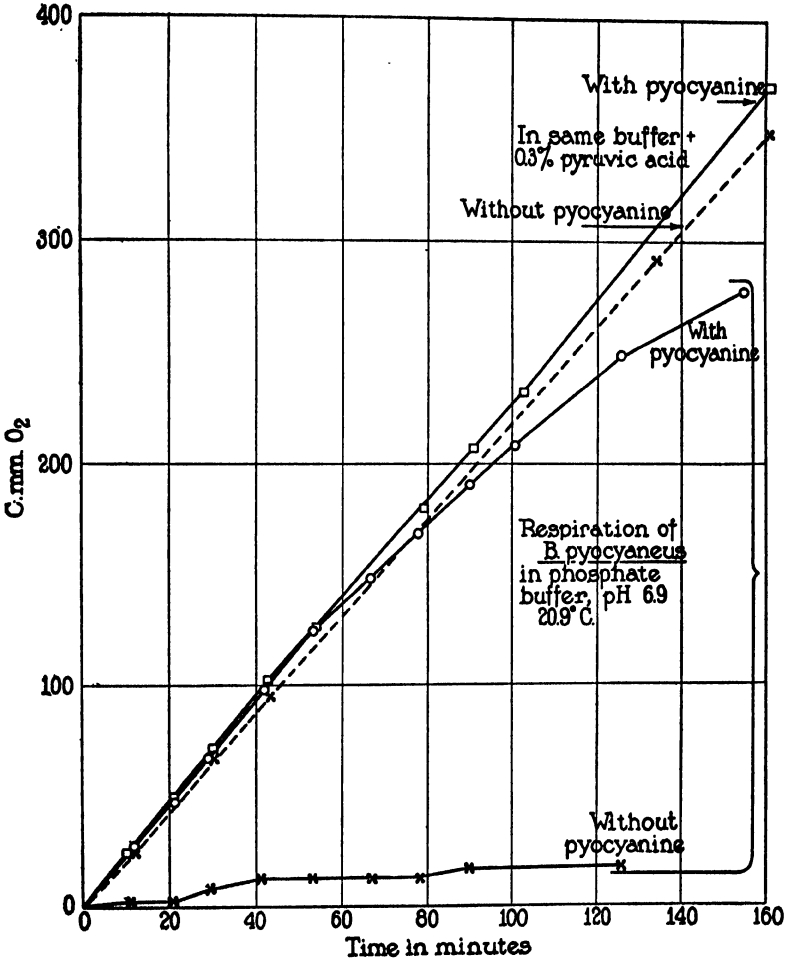

FIG. 4

be concluded that pyocyanine does not enhance respiration under all circumstances, but that it only catalyzes the oxidation of certain unknown substances closely associated with the bacteria, perhaps bacterial lipoids and polysaccharides, or certain products of their disintegration.

The Significance of Pyocyanine as a Respiratory Enzyme.—The fact that *B. pyocyaneus* has an appreciable oxidative metabolism even in the absence of pyocyanine tends to indicate that the latter does not play the main rôle in the respiration of the bacteria. Also further experiments show that pyocyanine is an accessory ferment, the effect of which is dependent upon the presence of one or several other respiratory ferments, amongst which must be considered the cytochrome shown spectroscopically by Yaoi and Tamiya (4) to be present in *B. pyocyaneus*. Their observation has been fully confirmed in the course of these experiments.

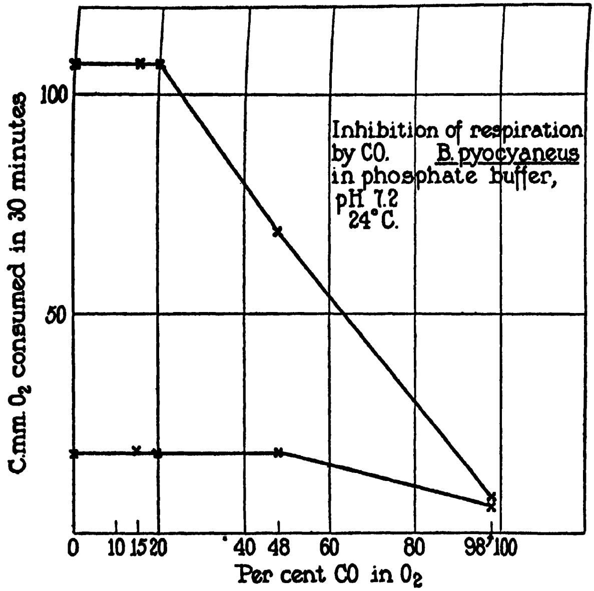

Fig. 5

In a test-tube experiment at pH > 5, the oxidation and reduction of leucopyocyanine is in no way impeded by carbon monoxide or by potassium cyanide. But, as shown in Figs. 5 and 6, these substances, known readily to form complexes with heavy metals, impede not only the basic respiration of the bacteria alone, but also the respiration due to pyocyanine. Figs. 5 and 6 further reveal the curious phenomenon that the pyocyanine enhanced respiration is affected at a lower potassium cyanide and carbon monoxide threshold-concentration than is the basic respiration. As the association of different hemochromogens, known as cytochrome, does not combine with carbon monoxide

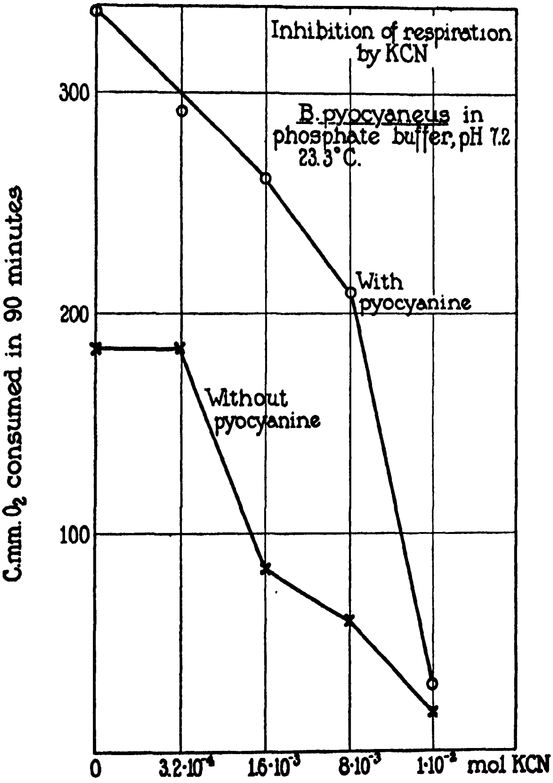

FIG. 6

(at physiological pH) the observation that the respiration of *B. pyocyaneus*, with and without pyocyanine, is impeded by carbon monoxide indicates that the cytochrome, although it may be a necessary link in the chain of bacterial oxidation catalysts, is not the final one, and that that chain is not complete without the carbon monoxide sensitive respiratory enzyme of Warburg.

The increase of respiration induced by pyocyanine is not specific for the species *B. pyocyaneus*. It has been observed in varying degree with other microorganisms containing cytochrome, *i.e.*, with staphylococci (Fig. 7) and pneumococci (Fig. 8); whereas with obligatory anaerobics such as *B. tetani*, which do not contain any cytochrome, pyocyanine had no effect. Furthermore, its effect is not restricted to bacteria only, but may be observed also with animal cells. Fig. 9 shows the increase in respiration induced by pyocyanine in red blood corpuscles of the rabbit.

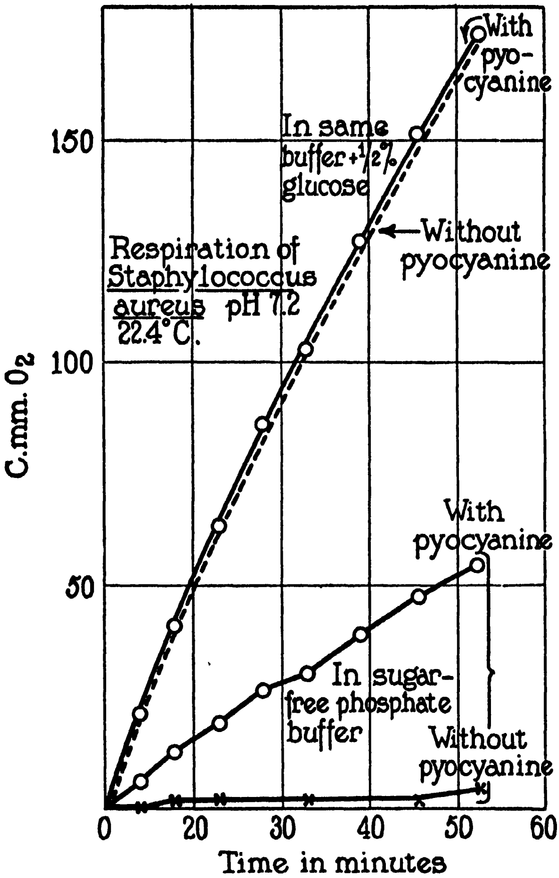

FIG. 7

The fact that pyocyanine is to a large degree not species-specific makes reasonable the view that the characteristic properties which condition its significance as an oxidation catalyst are of a purely physicochemical nature, the decisive feature being reversibility. If this is so one may expect that any other reversible system of the same potential range would have the same biological effect, granting that it has no other properties, such as solubility, permeating propensity,

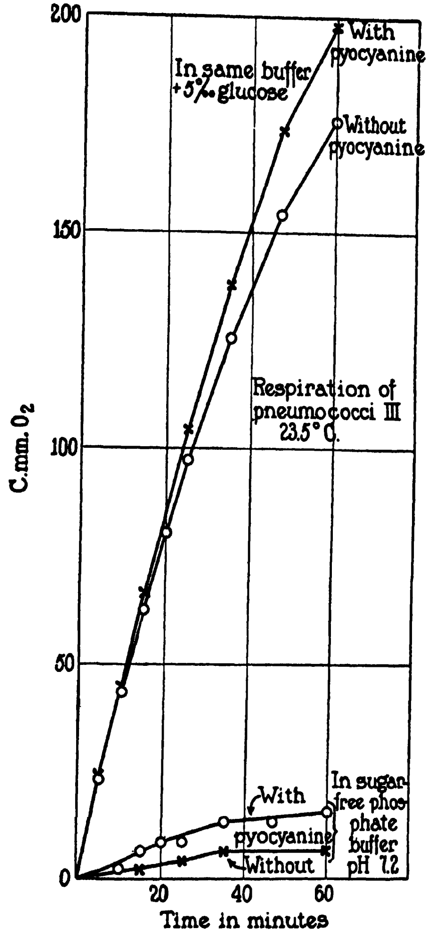

FIG. 8

toxicity, which might act as counteracting influences. Pyocyanine seems to be favored in respect to these secondary chemical properties by its low affinity for proteins and its high affinity for lipoids. Of the synthetic organic oxidation-reduction systems methylene blue and indigo tetrasulfonate are closest to pyocyanine in their potential range. At pH 7.0 and 30°C. the normal potentials are:

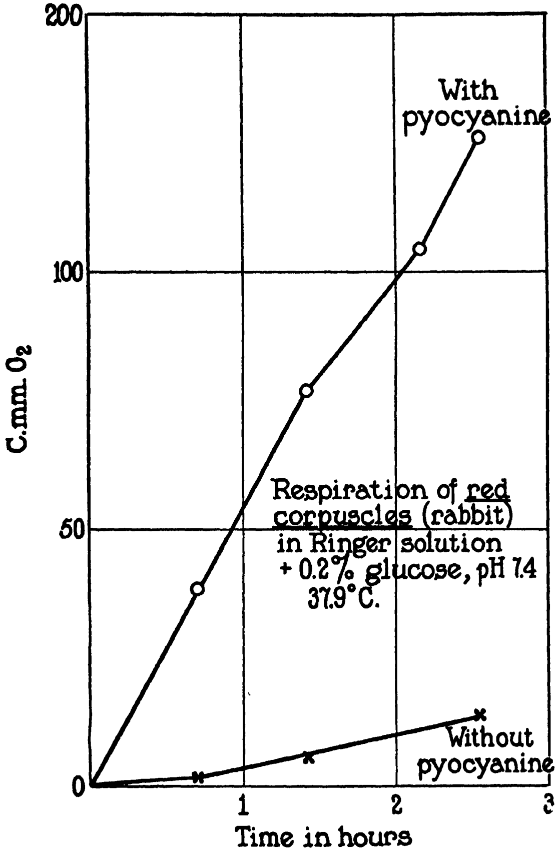

FIG. 9

	mv.
Indigotetrasulfonate	−46
Pyocyanine	−34
Methylene blue	+11

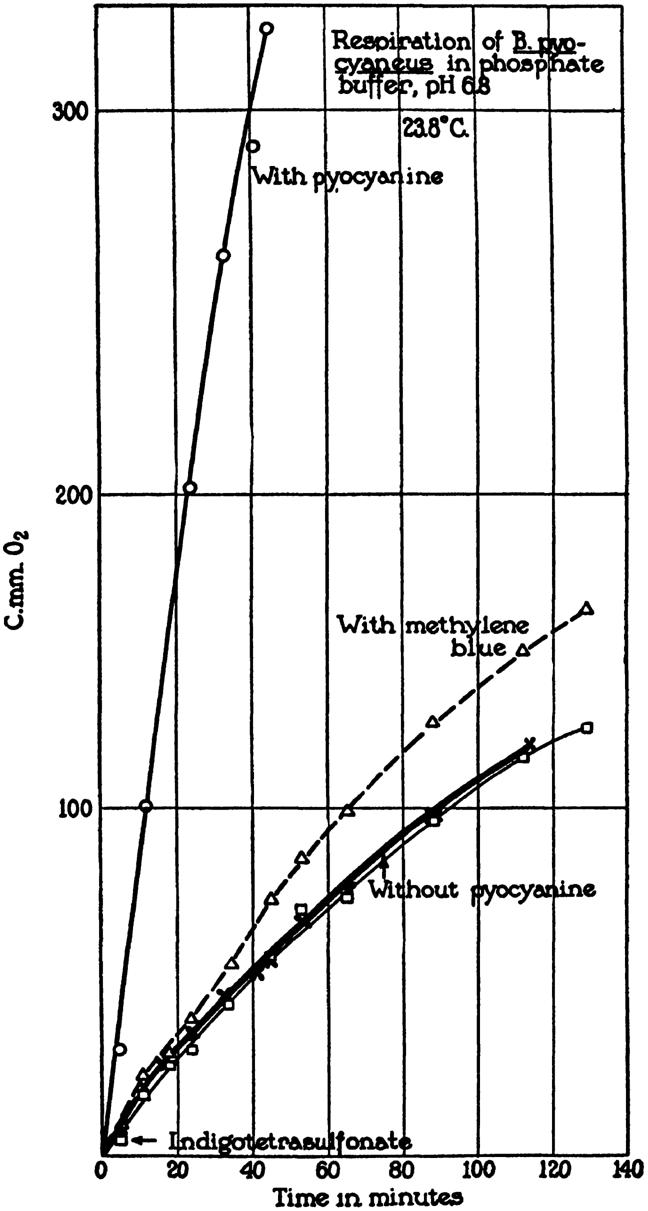
Respiration of B. pyocyaneus in phosphate buffer, pH 6.8
23.8°C.
With pyocyanine
With methylene blue
Without pyocyanine
Indigotetrasulfonate
C.mm. O_2
300
200
100
0
20
40
60
80
100
120
140
Time in minutes

FIG. 10

Fig. 10 shows the comparative effect exerted by these three substances in equal molecular concentrations $\left(\frac{\text{Mol}}{5{,}000}\right)$ on the respiration of *B. pyocyaneus*. Whereas indigotetrasulfonate with a potential range more negative than that of pyocyanine is devoid of effect the more positive methylene blue induces an increase of 30 per cent, and pyocyanine itself an increase of 440 per cent. In connection with the significance to be attributed to the value of the normal potential of the catalyst there is one obvious condition: It must be more positive than the reduction potential of the compound of which it catalyzes the oxidation. The findings just given would seem to indicate that the potential range of pyocyanine represents the lower limit of the potential range in which hydrogen can be accepted from the bacteria.

The experiment with methylene blue links this work with earlier observations showing that methylene blue enhances not only the respiration of acetone-treated yeast and staphylococci (Meyerhof (5)), but also the respiration of living cells as *e.g.* red and white blood cells, sea urchin and star fish eggs, and cancer cells (Barron and coworkers (6)). These observations disclosed the possibility of biological oxidations being catalyzed by autooxidizable hydrogen acceptors. The search for substances in the living cell which act like the artificial model, methylene blue, led to the isolation of two kinds of substances; First, a group having the character of reversible systems (echinochrome, hermidin) but not as yet proved to play an actual rôle as respiratory enzymes of the living cell; second, the respiratory supplement discovered by Michaelis and Salomon (7) in extracts of various organs, especially liver, which has a mode of action not yet shown to be that of a reversible system.

SUMMARY

Pyocyanine, the blue pigment of *B. pyocyaneus*, can increase the respiration of living cells to a great degree (maximum observed increase 24-fold). The reversibility of its oxidation and reduction is responsible for this. The effect is non-species-specific and has been observed in varying degrees with *B. pyocyaneus*, *Staphylococcus aureus*, Pneumococcus Type III, and the red blood corpuscles of rabbits.

The effect of pyocyanine is dependent on the presence of another

respiratory ferment sensitive to potassium cyanide and carbon monoxide.

The increase of respiration induced by pyocyanine is paralleled by an increase in the respiratory quotient. The pyocyanine catalysis is not indiscriminately effective in all oxidations, but only in the oxidation of certain substances closely associated with the bacterial body.

I wish to express my sincere thanks to Dr. L. Michaelis, in whose laboratory this work was carried out, for many helpful suggestions and criticisms.

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EDESTIN ALS SUBSTRAT FÜR DIE NEPHELOMETRISCHE BESTIMMUNG PROTEOLYTISCHER ENZYME

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(Eingegangen am 24. April 1931)

Den von *Rona* und *Kleinmann* für *Casein* (1), *Serum* (2) und *Serumalbumin* (3), von *Kleinmann* und *Stern* für *Gelatine* (4) und neuerdings von *Stern* für *Organproteine* (5) als Substrate für die nephelometrische Verfolgung proteolytischer Vorgänge ausgearbeiteten Methoden sei hier eine Arbeitsvorschrift für *Edestin* als weiteres Substrat angefügt.

Edestin, nach Vorschrift von *Osborne* aus Hanfsamen dargestellt, dient bekanntlich seit längerer Zeit zur Pepsinbestimmung für die Zwecke der Klinik. Die von *Fuld* und *Levison* (6) angegebene und weiterhin von *Ege* (7) modifizierte klinische Pepsinbestimmung beruht auf der Unlöslichkeit des unverdauten Edestins in neutraler Lösung. Um den Anforderungen der nephelometrischen Analyse zu genügen, die eine optische Vorrichtung von der Genauigkeit des *Kleinmann*schen Nephelometers (8)[1] (Ablesungsfehler ±1%) zur Voraussetzung hat, mußten die bei den genannten älteren Methoden als Fällungsreagenzien benutzten Neutralsalze durch ein Reagens ersetzt werden, daß eine hinreichend gleichförmige und stabile Trübung erzeugt. Als geeignet hierfür erweist sich *Sulfosalicylsäure*, die bereits für andere nephelometrische Methoden mit Erfolg benutzt wird.

Die Verfolgung proteolytischer Vorgänge, soweit sie sich auf die erste Phase des Abbaues nativer Eiweißkörper durch peptische, tryptische und katheptische Proteinasen erstrecken, mit Hilfe der nephelometrischen Analyse ist, wie kürzlich auch von *Grassmann* (9) anerkannt wurde, hinsichtlich der Abgrenzung des zu studierenden Prozesses den rein chemischen Methoden überlegen. Diese bestimmen titrimetrisch oder gasometrisch die durch die Enzymwirkung frei gelegten NH_2- oder COOH-Gruppen, die aus dem Substrat gleicherweise durch echte

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[1] *F. Schmidt* u. *Haensch*, Berlin, S. 42.

Proteinasen, wie auch durch Carboxy-Polypeptidasen und Dipeptidasen abgespalten werden. Bei Anwendung derartiger Methoden ist meist die präparative Zerlegung des vorliegenden Enzymgemisches mittels Adsorption und Elution erforderlich, um die beobachtete Wirkung auf bestimmte Proteasen zurückführen zu können. Bei nephelometrischer Analyse, bei der die Substratspaltung wie bei den anderen Methoden in homogener Lösung erfolgt, worauf das unverbrauchte Substrat mittels Zugabe geeigneter Trübungsreagenzien im Nephelometer bestimmt wird, ist eine solche Reinigungsvornahme unnötig, sofern man allein an der Wirksamkeit der vorhandenen echten Proteinasen interessiert ist, da sich die Tätigkeit der ereptischen Enzyme hierbei nicht störend bemerkbar macht.

Die Beschaffenheit der bis vor kurzem für die Nephelometrie zur Verfügung stehenden Substrate, von denen *Casein* und *Gelatine* als denaturierte Proteine von besonders leichter Angreifbarkeit und *Serum-albumin* infolge seiner ausgeprägten Resistenz gegen Tryptasen nicht als ideale Substrate für alle drei Gruppen der echten Proteinasen gelten können, machte es wünschenswert, weitere Methoden für native Eiweißkörper auszuarbeiten. Nachdem dies kürzlich vom Verfasser für gelöste *Organproteine* als Substrat tierischer Gewebsproteinasen durchgeführt wurde, soll im folgenden gezeigt werden, daß *Edestin* sich gleichmäßig als Substrat für das Studium *peptischer*, *tryptischer* und *katheptischer* Enzyme eignet.

Methodische Vorversuche

Herstellung der Edestinlösung

Als geeignet erwies sich eine nach der Vorschrift von *Fuld* und *Levison* bereitete 1°/₀₀ige Edestinlösung: 1 g Edestin, chemisch rein (Hersteller: *Pfanstihl & Co.*, Chicago), wird unter Erhitzen auf Siedetemperatur in 1000 ccm 0,03 n Salzsäure gelöst. Die Lösung wird nach Abfiltrieren restlicher Flöckchen unter Toluol im Kühlschrank aufbewahrt und die zum Versuch benötigte Menge jeweils durch ein quantitatives Filter zwecks Klärung filtriert. Es wird empfohlen, diese Lösung des öfteren frisch zu bereiten.

Fällungsreaktionen

Das von *Fuld* und *Levison* benutzte feste Natriumchlorid gibt eine für nephelometrische Zwecke unbrauchbare grobflockige Fällung. Die von *Ege* angegebenen Fällungsreagenzien (Ammonsulfat- und Natriumchloridlösung) erfordern zur Stabilisierung der auftretenden Trübung die Zugabe eines Schutzkolloids (Gummi arabicum), wodurch die Technik unnötig kompliziert wird. Salzsäure, die bei der nephelometrischen Serumalbuminmethode von *Rona* und *Kleinmann* zur Stabilisierung der mit Sulfosalicylsäure erzeugten Trübung dient, gibt bereits für sich allein mit Edestin eine Trübung, die jedoch rasch grobdispers wird.

Sulfosalicylsäure in 20%iger Lösung liefert bei passender Substrat- und Reagenzkonzentration eine ausreichend stabile und gleichförmige Trübung mit Edestin. Als obere Grenze wurde durch Ansetzung von Reihen eine Edestinmenge ermittelt, die 2 ccm der Edestinlösung in einem Gesamtvolumen von 22 ccm einschließlich der Sulfosalicylsäurezugabe entspricht. Unter Variierung der Reagenzmenge bei Einhaltung dieser Substratkonzentration ergab sich beim Vergleich im Nephelometer, daß bereits 1 ccm der Reagenzlösung zur quantitativen Erfassung der vorhandenen Proteinmenge ausreichend ist. Zwischen 1 und 5 ccm Reagenzzugabe findet keine Änderung der Trübungsintensität statt. Wird jedoch die Reagenzmenge weiter gesteigert, so beginnt die Intensität der Trübung langsam abzunehmen. Es wird daher künftig als bestimmt ausreichende, jedoch nicht übergroße, Reagenzmenge ein Volumen von 4 ccm der 20%igen Sulfosalicylsäurelösung gewählt.

Stabilität der Trübung. Die zum Zwecke der Analyse im Nephelometer erzeugten Trübungen zeigen erfahrungsgemäß einen mit der Beschaffenheit des Substrats, der Azidität der Lösung und der Art des zur Trübungserzeugung verwendeten Fällungsmittels wechselnden Gang. Gewöhnlich kann man bei fortlaufender Vergleichung der Trübungsintensität gegen einen festen Trübungsstandard im Nephelometer beobachten, daß kurz nach der Reagenzzugabe die Intensität der Trübung zunächst bis zu einem bestimmten Grenzwert steigt, der die völlige Erfassung des Substrats durch das Trübungsreagens anzeigt, sich sodann eine verschieden lange Periode hindurch, die im Interesse der Meßgenauigkeit möglichst nicht weniger als ½ Stunde betragen soll, auf dem gleichen Niveau hält, worauf dann unter makroskopisch sichtbarer Ausflockung eine Abnahme der Trübungsintensität infolge Ausbildung großer Aggregate einsetzt.

Da für diesen Versuch kein fester Standard nach *Kleinmann* (10) zur Verfügung stand, wurde auf eine von *Bechhold* und *Hebler* (11) angegebene Standardtrübung zurückgegriffen, die innerhalb der benötigten Versuchszeit von 90 Minuten als hinreichend stabil angesehen werden durfte. Sie besteht aus einer in Glycerin erzeugten Bariumsulfattrübung, die nach *Bechhold* Primärteilchen von durchschnittlich 2,5 μ Durchmesser aufweist und mindestens für 24 Stunden und höchstens für 8 Tage als stabil und gleichförmig betrachtet werden kann. Die gleichen Autoren haben auch die Bereitung eines Standard-$BaSO_4$-Sols in Glycerin-Isobutylalkohol angegeben, das gegen das erstgenannte, hier benutzte Sol geeicht wird und etwa 6 Monate brauchbar ist.

Die Standardtrübung wurde bereitet, indem m/5000 Hydroxylaminsulfat in Glycerin gelöst und mit der Lösung von m/5000 Bariumchlorid in Glycerin vermischt und auf ein Volumen von 75 ccm mit Glycerin aufgefüllt wurde. Die resultierende weißliche Trübung zeigte an dem Versuchstage keinerlei sichtbare Veränderung. Diese Lösung wurde in das linke Glas des *Kleinmann*schen Nephelometers als Standardtrübung eingefüllt und die Fensterhöhe für die stärkere der beiden untersuchten Edestintrübungen auf 20 mm und für die niedrigere Edestinkonzentration auf 15 mm fixiert.

Edestintrübung Nr. I. 2 ccm der 0,1%igen Edestinlösung + 16 ccm Aqua dest. + 4 ccm Sulfosalicylsäurelösung (20%ig). Sogleich nach Reagenzzugabe in das rechte Glas des Nephelometers eingefüllt. Standardtrübung links auf 20 mm fixiert.

Zeit nach Reagenzzugabe......	2	5	10	15	20	30	45	85 Minuten
Ablesung am rechten Fenster (Durchschn. mehrerer Beob.).	23	22,9	22,6	23	23,2	23	23	23 mm

Trotzdem die Edestintrübung nach 85 Minuten makroskopisch fein geflockt erschien, hatte sich der Intensitätswert der Trübung selbst nach dieser Zeitspanne noch nicht verändert. (Diese Beobachtung ist so zu deuten, daß auf eine gewisse Strecke die Abnahme der abgebeugten Lichtmenge infolge der Verringerung der Teilchenzahl kompensiert wird durch die Vergrößerung der reflektierenden Oberflächen.) Nach 45 Minuten war noch nicht die geringste makroskopisch sichtbare Veränderung eingetreten.

Edestintrübung Nr. II. 11 ccm der Edestinlösung + 17 ccm Aqua dest. + 4 ccm Sulfosalicylsäurelösung. Konzentration an Edestin demnach gleich 50% der Edestintrübung Nr. I. Standardtrübung links auf 15 mm fixiert.

Zeit nach Reagenzzugabe.....	2	5	10	15	20	30	50	90	Minuten
Abl. am rechten Fenster (Durchschnittswerte)......	34,8	34,2	34,5	34,5	34,1	34,6	34,3	34,5	mm

Hier war auch nach Verlauf von 90 Minuten noch nicht die geringste Flockung eingetreten. Es ist bemerkenswert, daß die bei anderen Substraten beobachtete anfängliche Zunahme der Trübungsintensität bis zu einem Grenzwert hier entfällt. Weit über die erforderliche Zeit hinaus ist die erzeugte Trübung stabil und gleichförmig, so daß die Messung der Trübung nach Beendigung von Fermentversuchen zu einem beliebigen Zeitpunkt, jedoch innerhalb der ersten Stunde nach Reagenzzugabe vorgenommen werden kann.

Proportionalität zwischen Trübung und Konzentration

Zur Prüfung dieser Frage wurde eine Reihe von sogenannten „Modellspaltungen" vorgenommen, bei denen der im wirklichen Fermentversuch beobachtete Ausschlag, der auf dem Verschwinden des Substrats unter dem Angriff des Enzyms beruht, ersetzt wird durch Variierung der Substratmengen unter Konstanthaltung aller übrigen Bedingungen.

Beispiel einer Modellspaltung

Spaltungsansatz. 5 ccm m/7 Veronalacetatgemisch + 12,4 ccm n/10 HCl + 20,5 ccm Edestinlösung (entsprechend etwa 7 ccm n/10 HCl) + Aqua dest. zu

46 ccm. Das p_H des Gemisches (Veronalacetat-HCl-Puffer nach *Michaelis* (12) beträgt etwa 2.

Auffüll-Lösung. Gleiche Zusammensetzung wie der Spaltungsansatz, jedoch an Stelle der Edestinlösung 20,5 ccm 0,03 n HCl.

Fermentlösung. Normaler Glycerinextrakt aus Kälbermilz nach *Waldschmidt-Leitz* und *Deutsch* (13), auf das dreißigfache Volumen mit Aqua dest. verdünnt. In dieser Verdünnung macht sich die Trübung, die das Eigeneiweiß des Extrakts mit Sulfosalicylsäure liefert, nicht mehr störend merkbar.

Analysengemische. Zur nephelometrischen Analyse wurden die unten beschriebenen „Spaltabnahmen“ im Volumen von je 5 ccm mit 13 ccm Aqua dest. und 4 ccm der Sulfosalicylsäurelösung versetzt und nach etwa 10 Minuten im Nephelometer gegen Abnahme Nr. 1 als Standard (Edestingehalt als 100% angesetzt) verglichen. Alle Bestimmungen wurden in Parallelen ausgeführt.

	1	2	3	4	5	6
	Edestin					
Spaltabnahmen:	100%	88,8%	77,7%	66,6%	55,5%	44,4%
Spaltungsansatz	4,5 ccm	4,0 ccm	3,5 ccm	3,0 ccm	2,5 ccm	2,0 ccm
Auffüllösung	0 „	0,5 „	1,0 „	1,5 „	2,0 „	2,5 „
Fermentlösung	0,5 „	0,5 „	0,5 „	0,5 „	0,5 „	0,5 „
Volumen:	5,0 ccm	5,0 ccm	5,0 ccm	5,0 ccm	5,0 ccm	5,0 ccm
Durchschnittswerte der Ablesungen im Nephelometer a)	*20 mm*	22,1 mm	25,4 mm	28,0 mm	35,7 mm	
b)	20,2 „	22,5 „	25,1 „	28,0 „	35,3 „	
a)	*15* „					30,5 mm
b)						31,0 „
Mittelwert:		22,3 mm	25,2 mm	28,0 mm	35,5 mm	30,7 mm
Scheinbare Substratspaltung						
berechnet		11,2%	22,3%	33,4%	44,5%	55,6%
gefunden		10,3%	20,7%	28,6%	43,7%	51,2%

Wie auch die folgende graphische Darstellung zeigt, ist die Übereinstimmung zwischen den vorhandenen und wiedergefundenen Substratmengen bei Anwendung der üblichen Formel für die Ermittlung der Konzentration aus der Trübungsintensität so befriedigend, daß auf die Konstruktion einer empirischen Eichkurve verzichtet werden kann, wenn es weniger auf die Ermittlung absoluter Spaltungsgrößen als auf die Vergleichung von enzymatischen Aktivitäten ankommt.

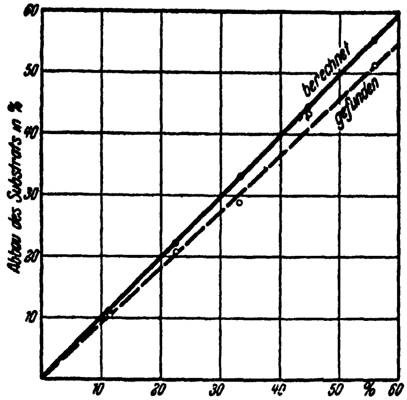

Abb. 1. Graphische Darstellung der berechneten und der experimentell gefundenen Substratspaltungen eines Modellversuchs.

Nachstehend wird an je einem Beispiel die Anordnung für Anstellung von Spaltversuchen mit *Pepsin*, *Trypsin* und *Kathepsin* erläutert:

Pepsin

Aufgabe. Ermittlung des zeitlichen Verlaufs des Abbaues von Edestin durch Pepsin bei optimalem p_H.

Versuchsanordnung. In einen Meßzylinder von 50 ccm Volumen werden 5 ccm m/7 Veronal-Acetatlösung + 18 ccm n/10 HCl + 20 ccm Edestinlösung 0,1%ig), entsprechend 7 ccm n/10 HCl + 3 ccm Aqua dest. einpipettiert. Volumen: 46 ccm, p_H 1,5 (optimal für die benutzte Pepsinlösung). Nach Mischen werden 10 ccm für zwei Nullabnahmen zu je 4,5 ccm entnommen, der restliche 1 ccm verworfen. Zu den verbliebenen 36 ccm des Spaltansatzes werden nach Vorwärmen im Wasserthermostaten bei 36°C unter Markieren der Zeit 4 ccm der Pepsinlösung zugegeben. Hier wurde z. B. Kaninchenmagenglycerinextrakt in 50facher Verdünnung benutzt. Nach Ablauf von je 5 Minuten werden dem Gemisch mit der Pipette 5 ccm entnommen und zur Unterbrechung der Enzymwirkung in 5 ccm 0,05 n NaOH einfließen gelassen. Nach der siebenten Abnahme verbleiben im Meßzylinder etwa 4 ccm, in denen bequem p_H gemessen werden kann. Die Abnahmen sind bei allen Methoden derart bemessen, daß sich in jeder Probe zu Beginn der Spaltung 2 mg Edestin befinden.

Nunmehr werden zu den Nullabnahmen, deren Eiweißgehalt gleich 100% gesetzt wird, je 0,5 ccm der Enzymlösung und unmittelbar darauf ebenfalls 5 ccm 0,05 n NaOH gegeben. Sodann werden zu allen Proben je 8 ccm Aqua dest. + 4 ccm Sulfosalicylsäurelösung (20%ig) pipettiert.

(Als Arbeitshilfe für rasches Pipettieren der Reagenzien hat sich im hiesigen

Laboratorium die Benutzung von *Luer*schen Glasinjektionsspritzen bewährt. An Stelle der Kanüle wird eine rechtwinklig gebogene dünne Glasröhre mittels einer Schliffverbindung aufgesetzt. Die zu pipettierenden Flüssigkeitsmengen können so mühelos bei horizontaler Spritzenhaltung abgemessen werden.)

Ergebnis der Analyse am Nephelometer

Zeit der Entnahme nach Versuchsbeginn:	0 Min.	5 Min.	10 Min.	15 Min.
Durchschnittliche Ablesung	*20 mm* *15 „*	21,8 mm	24,8 mm	30,5 mm
Substratspaltung		8,3%	19,4%	34,4%
Zeit der Entnahme nach Versuchsbeginn:	**20 Min.**	**25 Min.**	**30 Min.**	**35 Min.**
Durchschnittliche Ablesung	36,3 mm	41,8 mm	34,2 mm	38,4 mm
Substratspaltung	44,9%	52,1%	56%	60,9%

Das Ergebnis ist in der folgenden Abbildung graphisch dargestellt.

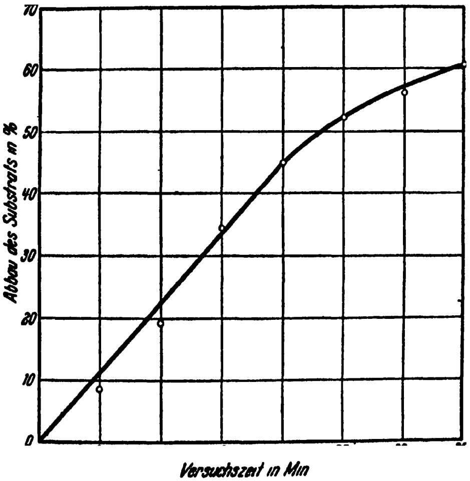

ABB. 2. Zeitlicher Verlauf der Edestinaufspaltung durch Pepsin (50 fach verdünnter Kaninchenmagenextrakt). p_H 1,5.

Trypsin

Aufgabe. Ermittlung des zeitlichen Verlaufs des Abbaues von Edestin durch Trypsin bei optimalem p_H.

Versuchsanordnung. Gleichartig wie unter „Pepsin“ beschrieben, jedoch zur Einstellung des optimalen p_H von etwa 8 an Stelle der HCl 4 ccm n/10 NaOH im Ansatz untergebracht. Als Trypsinlösung wurde Trypsin „Fairchild“ in einer Verdünnung von 1 : 30000 benutzt. Die Spaltung wurde wiederum in Wasserthermostaten bei 36°C vorgenommen. Die Intervalle der Probeentnahmen betrugen in diesem Beispiel je 10 Minuten. Zur Unterbrechung der Enzymwirkung wurden die Proben in 1,5 ccm n/10 HCl einfließen gelassen. Für die nephelometrische Analyse wurden zunächst den Nullabnahmen je 0,5 ccm der Fermentlösung + 1,5 ccm n/10 HCl zugefügt, sodann in alle Proben je 11,5 ccm Aqua dest. + 4 ccm Sulfosalicylsäure pipettiert. Der Spaltungsansatz war bei dem gewählten p_H von etwa 8 zunächst, getrübt, vor der Probeentnahme muß daher gut gemischt werden. Im Verlaufe des Spaltversuchs klärt sich der Ansatz rasch infolge des Verschwindens des Substrats.

Ergebnis der Analyse am Nephelometer

Zeit der Entnahme nach Versuchsbeginn	0 Min.	10 Min.	20 Min.	30 Min.	40 Min.	50 Min.	60 Min.
Durchschnittl. Ablesung	*20 mm* *15 „*	28,2 mm	32,6 mm	37,5 mm	32 mm	36,2 mm	41,5 mm
Substratspaltung		29,1%	38,7%	46,7%	53,1%	58,6%	63,9%

Die erhaltene Spaltungskurve zeigt die nachstehende Abbildung.

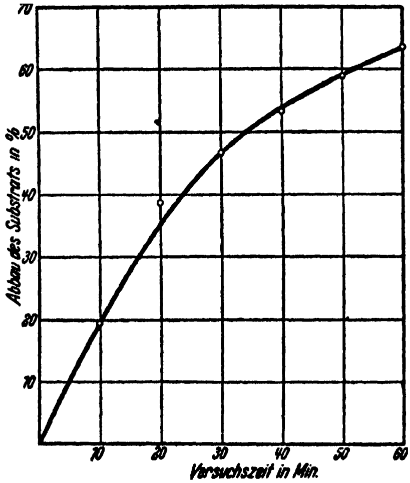

Abb. 3. Zeitlicher Verlauf der Edestinspaltung durch Trypsin. (Trypsin „Fairchild“ 1 : 30 000). p_H etwa 8.

Kathepsin

Aufgabe. Ermittlung der Aktivitäts-p_H-Kurve des Kathepsins der Kälbermilz unter Verwendung von Edestin als Substrat.

Versuchsanordnung. In eine Reihe von Meßzylindern von je 50 ccm Fassungsvermögen werden je 5 ccm m/7 Veronal-Acetatlösung, ferner variierende Mengen von n/10 HCl bzw. n/10 NaOH zur Einstellung des gewünschten p_H, weiterhin je 20 ccm der 0,1%igen Edestinlösung (entsprechend etwa 7 ccm n/10 HCl) pipettiert und mit Aqua dest. zu 46 ccm Volumen aufgefüllt. Jedem Ansatz werden 10 ccm für zwei Nullabnahmen zu je 4,5 ccm entnommen, der restliche 1 ccm verworfen. Zu den verbliebenen 36 ccm werden 4 ccm der Fermentlösung gegeben. (In diesem Beispiel auf das dreißigfache Volumen mit Aqua dest. verdünnter Normalglycerinextrakt aus Kälbermilz (14); in dieser Verdünnung ist ein störender Einfluß durch das Eigeneiweiß des Extrakts ausgeschaltet.)

Die Zylinder wurden nach Zugabe von Toluol mit Kautschukstopfen verschlossen bei 37°C im Brutschrank gehalten; auch die Nullabnahmen wurden gleichartig behandelt. Die Fermentlösung wurde im Kühlschrank aufbewahrt.

Nach Verlauf von 18 und 42 Stunden wurden den Spaltgemischen je 5 ccm entnommen. Im p_H-Bereich von etwa 4 bis 7 ist das Edestin schwer löslich und bildet Trübungen oder Flocken in den Spaltgemischen. Es muß deshalb vor jeder Probenahme gut mit der Pipette gemischt werden, worauf sich die Flocken leicht verteilen lassen. Der Flockungsbereich ist bei Edestin, verglichen mit Casein, nach der alkalischen Seite zu verbreitert. Die Analyse selbst wird jedoch davon nicht berührt, da das ausgeflockte Substrat vor der Reagenzzugabe durch passend gewählte Salzsäuremengen wieder in klare Lösung gebracht wird. Die in diesem Beispiel hierfür erforderlichen Säuremengen sind unten aufgeführt. Ein Überschuß an Säure ist möglichst zu vermeiden, da die mit Sulfosalicylsäure erzeugte Trübung sonst weniger stabil wird. Da die Auflösung des ausgefallenen Edestins durch die ausreichende Säuremenge einige Zeit (bis zu 15 Minuten) erfordern kann, sind vor weiterer Säurezugabe immer einige Minuten abzuwarten. Tritt trotzdem bei derartigen Ansätzen während der Messung eine makroskopisch sichtbare Vergröberung der Dispersität ein, so wird davon, wie unter den methodischen Vorversuchen (S. 368) gezeigt wurde, das Ergebnis nicht wesentlich beeinflußt.

Für jedes der beiden Probeentnahmeintervalle wird eine der bei gleicher Temperatur, jedoch ohne Ferment, aufgehobenen Nullabnahmen als Standard verwendet, nachdem kurz vor der Analyse 0,5 ccm der Fermentlösung und die gleiche Menge Salzsäure zugegeben wurde, die bei der Spaltabnahme zur Auflösung eventuell ausgeflockten Substrats gedient hatte.

Das Volumen aller Proben wird zur Analyse mit Aqua dest. auf 18 ccm aufgefüllt und nach Zugabe von 4 ccm Sulfosalicylsäure möglichst anschließend nephelometriert.

In dem in den Zylindern verbliebenen Rest wird p_H gemessen.

Beispiel

Nr.	Im Ansatz untergebracht	Aussehen	p_H	Zur Analyse	Spaltung nach	
					18 Std.	42 Std
1	18 ccm n/10 HCl	klar	1,46	—	0 %	0 %
2	14 „ n/10 „	„	1,6	—	0 %	0 %
3	12 „ n/10 „	„	1,7	—	0 %	5 %
4	9 „ n/10 „	„	2,1	—	0 %	7 %
5	6 „ n/10 „	trübe	*3,4*	*5 ccm n/100 HCl*	*21,6%*	*27,8%*
6	2,4 „ n/10 „	flockig	4,55	6 „ n/10 HCl	16,7%	24,5%
7	5 „ n/100 NaOH	„	6,17	7 „ n/10 HCl	0 %	7 %

Das Optimum der Enzymwirkung lag hier demnach etwa bei p_H 3,5. Die erhaltenen Kurven sind in der folgenden Abbildung wiedergegeben.

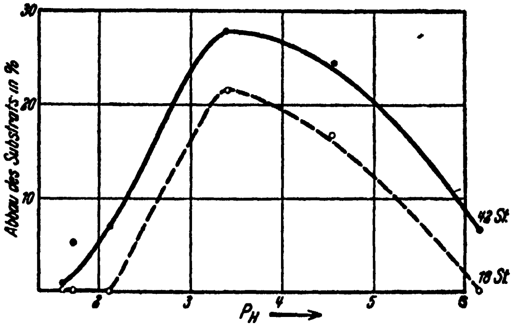

Abb. 4. Aktivitäts-p_H-Kurve von Kathepsin gegenüber Edestin. (30fach verdünnter Glycerinextrakt aus Kälbermilz.)

Aus den angeführten Beispielen geht hervor, daß Edestin als Substrat gleicherweise für die Untersuchung *peptischer*, *tryptischer* und *katheptischer* Spaltungen mittels nephelometrischer Analyse brauchbar ist.

ZUSAMMENFASSUNG

1. Es werden die Gründe erörtert, aus denen die Heranziehung weiterer Proteine als Substrate für die Verfolgung proteolytischer Vorgänge auf nephelometrischem Wege erwünscht erscheint.

2. Zu den kürzlich vom Verfasser herangezogenen tierischen Organproteinen wird hier als weiteres natives Protein *Edestin* aus Hanfsamen

nutzbar gemacht. Die älteren, vorwiegend für klinische Zwecke gedachten Methoden von *Fuld* und *Levison*, sowie von *Ege*, die ebenfalls mit Edestin als Substrat für Pepsinbestimmung arbeiten, mußten für die Zwecke der Nephelometrie völlig umgestaltet werden. Als Trübungsreagens wird *Sulfosalicylsäure* verwendet. Die unter Einhaltung der ausführlich gegebenen Arbeitsvorschrift erzeugte Trübungsreaktion ist, wie gezeigt wird, reproduzierbar, stabil und ergibt befriedigende Proportionalität zwischen Konzentration an Protein und Trübungsintensität im *Kleinmann*schen Nephelometer.

3. An Versuchsbeispielen wird belegt, daß Edestin als Substrat für die Untersuchung von *Pepsin*, *Trypsin* und *Kathepsin* geeignet ist, wodurch ein Vergleich der enzymatischen Leistung der verschiedenen Proteinasen gegenüber dem gleichen Substrat ermöglicht wird.

4. Die ausgearbeitete Methodik ist empfindlich, exakt und gestattet rasche Durchführung größerer Versuchsreihen. In jeder Analysenprobe befinden sich bei Versuchsbeginn 2 mg Edestin, deren enzymatischer Abbau mit einer Fehlerbreite von ±5 bis 10%, je nach der Größe der Ausschläge, verfolgt werden kann.

Herrn Professor Dr. *Michaelis* sei für sein stetes Interesse bei der Durchführung der Arbeit auch an dieser Stelle bestens gedankt.

Herrn Geheimrat Professor Dr. *C. Duisberg*, der die Durchführung der Versuche durch Verleihung eines Auslandsstipendiums ermöglichte, danke ich aufs beste.

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ÜBER DIE TRYPTASE FARBLOSER BLUTZELLEN

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(Der Redaktion zugegangen am 5. Mai 1931)

ALLGEMEINER TEIL

Die vorliegende Untersuchung wurde ausgeführt, um weiteres experimentelles Material zur Frage des Vorkommens von Tryptase in farblosen Blutzellen zu erhalten. Dies schien erwünscht im Hinblick auf Widersprüche und Unklarheiten in den Arbeiten, die sich mit dem proteolytischen System der Leukocyten beschäftigen.

Nach den grundlegenden Untersuchungen von Opie1) und Jobling2) haben sich u. a. Müller3), Jochmann und Lockemann4), Fiessinger5), ferner in jüngster Zeit Willstätter und Bamann6), Kleinmann und Stern7) und Ölkers und Fischgold8) mit diesem Gegenstande beschäftigt. Ohne den inzwischen von Kleinmann und Mitarbeitern an den einzelnen Blutbestandteilen durchgeführten Untersuchungen irgendwie vorgreifen zu wollen, sei über das Ergebnis eigener Versuche an einem Objekt, nämlich an den sterilen Exsudatleukocyten des Meerschweinchens, berichtet.

Willstätter,Bamann und Rohdewald6) bestätigten (in der 2. und 5. Abhandlung über Enzyme der Leukocyten) die Beobachtung von Opie, daß in den farblosen Blutzellen, und zwar sowohl der Pflanzenfresser wie der Fleischfresser, neben dem im schwach sauren Gebiet optimal wirksamen Kathepsin, das mit der ubiquitären Gewebsproteinase identisch ist, noch eine zweite echte Protease vorhanden sei, die optimal bei p_H 8 wirke und sowohl durch ihre Spezifität wie ihr Verhalten gegen Enterokinase als tryptische Proteinase gekennzeichnet sei. Im Anschluß daran wird von diesen Autoren die Frage aufgeworfen, ob nicht vielleicht diese Leukotryptase die Enzymquelle der Pankreasdrüse darstelle, die sie nach einem Aussonderungsprozeß in den Verdauungskanal sezerniere. Da bei ihren Versuchen mit einem natürlichen Leukocytengemisch gearbeitet wurde, wie es bei Sedimentation oder nach dem Szilárdschen Verfahren aus Blut erhalten wird, konnte die Behauptung von Opie, wonach die katheptische Proteinase lymphocytären und die tryptische myeloischen Ursprungs sei, nicht nachgeprüft werden.

Kleinmann und Stern7) (S. 99ff.) fanden in Glycerinauszügen von Rindermischleukocyten, die nach Szilárd9) gewonnen waren, gegenüber Casein und

Serumalbumin als Substrat bei Anwendung der nephelomet[illegible]ntersuchungsform allein Kathepsin. Da bei der nephelometrischen Analyse allein die Wirksamkeit echter Proteinasen zur Beobachtung gelangt, konnte auf Grund dieses Befundes vermutet werden, daß die von den anderen Autoren beobachtete tryptische Wirkung ebenso wie wahrscheinlich die α-Lienoprotease Hedins[10]) nur vorgetäuscht sei durch die Tätigkeit der bei p_H 8 optimal wirksamen ereptischen Enzyme (Poly- und Dipeptidase), die bei der Bestimmungsform der Titration freigelegter COOH-oder NH_2-Gruppen nicht von echten Proteinasen unterscheidbar sind. Dem stand jedoch entgegen, daß Willstätter, Bamann und Rohdewald[6]) (in der 5. Abhandlung über die Enzyme der Leukocyten, S. 120) die leukocytäre Tryptase durch Darmenterokinase aktivierbar fanden. Der beobachtete Aktivierungseffekt war allerdings nur relativ schwach, wurde unter gleichen Versuchsbedingungen öfters vermißt und trat ausschließlich in Glycerinsuspensionen (nicht Autolysaten, Extrakten usw.) auf, in denen auch eine Selbstaktivierung der tryptischen Wirkung von oft größerem Ausmaß eintrat. So erschien dieser wesentlichste Punkt experimentell nicht genügend gesichert.

Die von Ölkers und Fischgold[8]) letzthin publizierten Befunde an menschlichen Leukocyten erscheinen zur Beantwortung der Frage nach einer echten Tryptase nicht geeignet. Ohne p_H-Aktivitätskurven über das ganze physiologisch wichtige Gebiet durchzuführen, beschränkten sie sich auf den Nachweis einer Aufspaltung von Gelatine bei p_H 8 mittels der Titration der NH_2-Gruppen nach Linderström-Lang. Abgesehen davon, daß Gelatine infolge der häufig beobachteten teilweisen Angreifbarkeit durch niedere Proteasen kein geeignetes Substrat für den Nachweis echter Proteinasen darstellt, würden sie z. B. in Milzauszügen hierbei ebenfalls eine Tryptase (die α-Protease Hedins) finden, von der Waldschmidt-Leitz und Deutsch[11]) und dann Kleinmann und Stern[7]) auf verschiedene Weise gezeigt haben, daß sie mindestens zum großen Teil durch ein Zusammenwirken der bei p_H 8 noch schwach wirksamen katheptischen Proteinase mit den bei dieser Reaktion optimal wirksamen größeren Ereptasemengen vorgetäuscht ist.*)

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*) Anm. b. d. Korrektur: Versuche mit alkalischen und sauren, wäßrigen wie glycerinhaltigen Organextrakten, die in letzter Zeit von Kleinmann u. Mitarbeiter (unveröffentlicht) durchgeführt wurden, haben doch wieder die Möglichkeit nähergerückt, daß ein gewisser Anteil der von Hedin gefundenen tryptischen Effekte relativ kleinen Tryptasemengen zugeschrieben werden muß, die in wechselnder Konzentration im Gewebe vorkommen und die gegenwärtig darauf untersucht werden, ob sie in der Tat cellulären Ursprungs sind, oder ob sie aus den im Blut und Gewebe enthaltenen Leukocyten stammen. (Erschienen in der Biochem. Z.).

Zur Entscheidung der Frage stellten wir uns zur Aufgabe, mittels der nephelometrischen Methodik nach der Tryptase in einem Untersuchungsobjekt zu fahnden, in dem nach den vorhandenen Literaturangaben eine solche am reichlichsten zu finden sein muß, nämlich in frischen Exsudatleukocyten.

Von einer größeren Reihe erwachsener Meerschweinchen wurde nach der von De Haan[12]) im Hamburgerschen Institut ausgearbeiteten Technik mittels Injektion steriler physiologischer Kochsalzlösung in die Bauchhöhle nach 3–5stündiger Verweilzeit ein Exsudat gewonnen, das überwiegend polynucleäre, neutrophile Leukocyten enthielt.

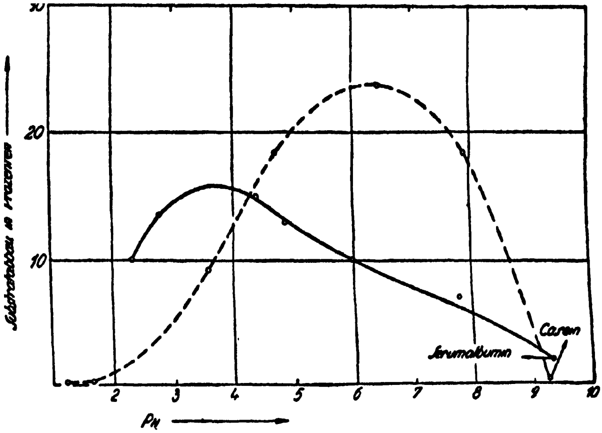

FIG. 1. Aktivitäts-p_H-Kurven von Leukocytenauszügen bei Verwendung von Casein und Serumalbumin als Substrat.

Diese sollen nach Opie[1]) die Träger der Tryptase darstellen. Die aus diesen Leukocyten hergestellten essigsauren Glycerinauszüge wurden in ihrem Verhalten gegen ihr in die Auszüge übergegangenes Eigeneiweiß, ferner gegen Edestin, Casein und Serumalbumin über das physiologische p_H-Gebiet hinweg nephelometrisch geprüft. Unter Bestätigung der Befunde an Rindermischleukocyten von Kleinmann und Stern wird bei Verwendung von Casein und Serumalbumin als Substrat nur ein im schwach sauren Gebiet liegendes Optimum gefunden, und zwar finden wir es für Serumalbumin bei p_H etwa 4 und für Casein bei p_H 6,5.

Wenn hingegen die vom Verfasser kürzlich in die nephelometrische Technik eingeführten Substrate, nämlich das in die Extrakte übergegangene Eigeneiweiß[13]) der Leukocyten und weiterhin Edestin[14]), zum Nachweis der proteolytischen Wirksamkeit herangezogen werden, zeigt die Aktivitäts p_H Kurve in beiden Fällen zwei deutlich ausgeprägte Maxima, das erste bei p_H 4 und das zweite bei 8. Während die enzymatische Leistung gegenüber dem Eigeneiweiß bei beiden Enzymen die gleiche ist, ist für Edestin die tryptische Wirksamkeit größer als die katheptische.

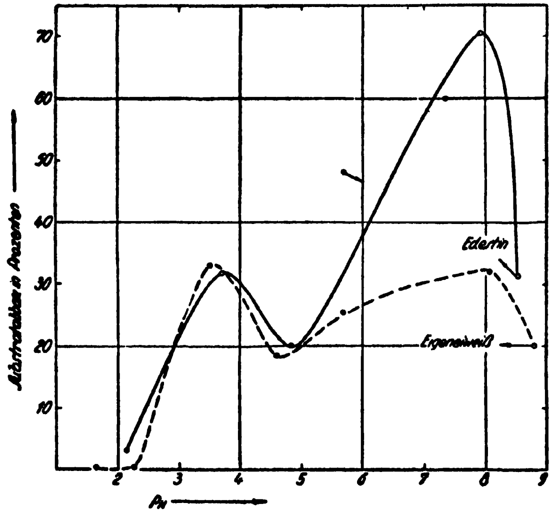

FIG. 2. Aktivitäts-p_H-Kurven der Leukocytenauszüge bei Verwendung von Eigeneiweiß der Extrakte und von Edestin als Substrat.

Zum Vergleich seien die entsprechenden Kurven für den Leberextrakt des Meerschweinchens gebracht, die ein tryptisches Optimum vermissen lassen. Auch in der Niere des Meerschweinchens finden wir bei Verwendung von Eigeneiweiß nur ein katheptisches Optimum bei p_H 4.*)

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Im Angesicht der an nativen Proteinen (Eigeneiweiß und Edestin) und mittels einer Technik gewonnenen Ergebnisse, die jede Störung durch ereptische Enzyme ausschließt, ist an dem Vorhandensein einer echten tryptischen Proteinase in den untersuchten Leukocyten nicht mehr zu zweifeln. Die abweichenden Resultate an Casein und Serumalbumin sind derart zu deuten, daß bei dem letzteren infolge seiner bekannten Resistenz gegen Trypsin nur das katheptische Spaltungsoptimum gefunden wird. Das für Casein bei p_H 6–7 gelegene Optimum ist als Mischoptimum der Kathepsin- und der Tryptasewirkung anzusehen. Dies stellt eine Parallele zu dem von Dernby[15]) bei der Hefeautolyse gefundenen Mischoptimum dar.

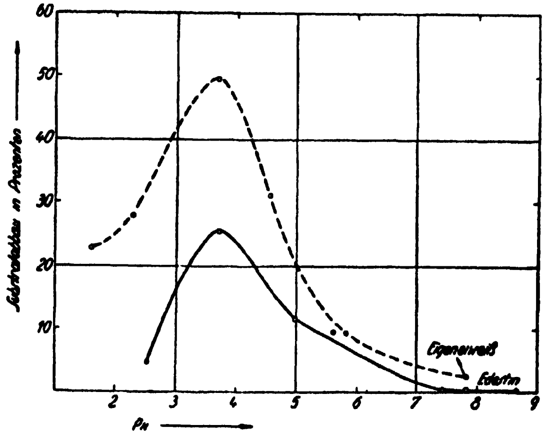

FIG. 3. Aktivitäts-p_H-Kurven des Leberextraktes gegenüber Eigeneiweiß und Edestin als Substrat.

Da die verwendeten Exsudatleukocyten neben den Polynucleären auch Mononucleäre (lymphatische Leukocyten) enthielten, kann nicht mit Sicherheit ausgesagt werden, wie sich die beiden gefundenen Proteinasen auf die einzelnen Kategorien verteilen. Falls die Tryptase auf die myeloischen Leukocyten beschränkt sein sollte, wie Opie annimmt, so betrachten wir es auf der anderen Seite als sehr wahrscheinlich, daß die katheptische Proteinase in beiden Formen gleichmäßig vertreten ist, da bisher noch keine tierische Zelle gefunden wurde, die frei von Kathepsin ist.

Die Frage der Aktivierbarkeit der leukocytären Tryptase durch

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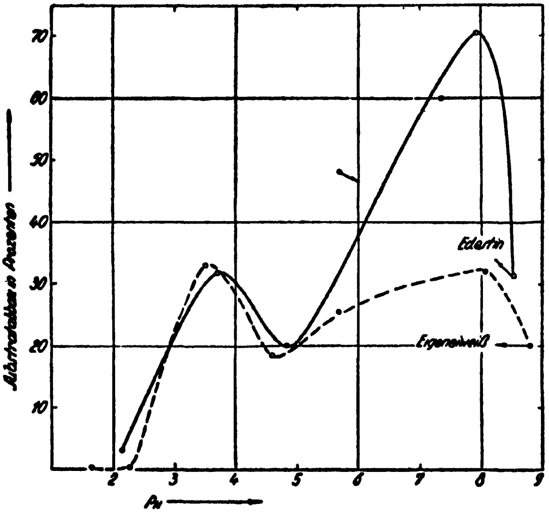

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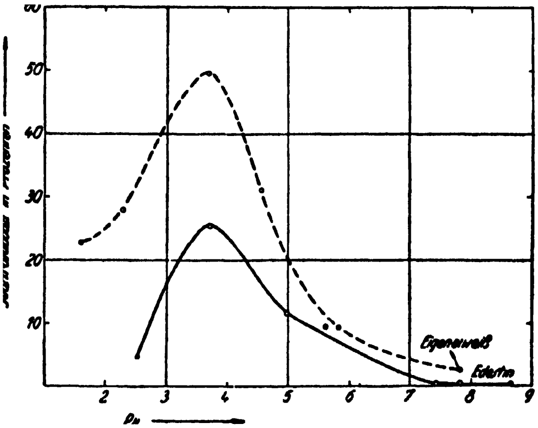

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Die Frage der Aktivierbarkeit der leukocytären Tryptase durch

Kinase wurde unter Heranziehung von Schweine- und von Meerschweinchenkinasepräparaten nochmals geprüft. Bei Verwendung von Edestin als Substrat konnte in Übereinstimmung mit Willstätter, Bamann und Rohdewald keine Aktivierung der in den Extrakten vorliegenden Tryptase mittels der Kinaselösungen beobachtet werden, deren Wirksamkeit zuvor an Pankreastryptase des Meerschweinchens geprüft worden war. Im Gegenteil wurde wiederholt gefunden, daß die in den gereinigten Kinaselösungen noch verbliebenen kleinen Trypsinmengen durch die Leukocytenauszüge in ihrer Wirksamkeit gegen Edestin gehemmt wurden. Auch an einer Suspension der Leukocyten, die nach den genannten Autoren gelegentlich durch Kinase aktivierbar sein sollte, wurde der gleiche negative Befund erhoben.

TABELLE 1

Vergleich der Aktivität der Leukocyten- mit der der Pankreastryptase des Meerschweinchens gegenüber dem Eigeneiweiß unter dem Einfluß von verschiedenen Schwermetallen und Selenit

Die Aktivität der unbeeinflußten Enzyme wird zu 100% angenommen

mg/ccm	Kupfer $7 \cdot 10^{-2}$	Zink $8 \cdot 10^{-2}$	Quecksilber $14 \cdot 10^{-2}$	Mangan $11 \cdot 10^{-2}$	Eisen $7 \cdot 10^{-2}$	Selenit $25 \cdot 10^{-2}$
Pankreastrypsin:	53,5%	14%	150%	67%	156%	112%
Leukocytentrypsin:	21 %	0%	167%	195%	94%	167%

Die mangelnde Aktivierbarkeit der leukocytären Tryptase kann darauf zurückzuführen sein, daß entweder der Tryptasekomplex bereits im vollaktivierten Zustande vorliegt, oder darauf, daß diese Tryptase überhaupt nicht durch die verwendete artfremde und arteigene Kinase aktivierbar ist. Nach Waldschmidt-Leitz[16]) kann bereits vollaktivierte Tryptase durch Aluminiumhydroxyd nach Willstätter wieder in das inaktive Trypsin und in die Kinase zerlegt werden. Ein mit leukocytärem Trypsin angestellter Trennungsversuch verlief jedoch negativ: Die nach der Adsorption verbliebene Restlösung enthielt fast die gesamte Tryptase, die sich durch Kinase nicht aktivieren ließ.

Weiterhin wurde das Verhalten der Leukocyten- und der Pankreastryptase des Meerschweinchens gegen die gleichen Konzentrationen verschiedener Schwermetall- und Selenitionen unter Verwendung des

Eigenproteins als Substrat geprüft. Diese Methode hatte sich dem Verfasser bei der Unterscheidung der katheptischen Proteinasen verschiedener Spezies und verschiedener Organe der gleichen Spezies bewährt.[13]) Beide Tryptasen werden durch Kupfer und Zinkionen beträchtlich gehemmt, durch Quecksilberionen erheblich aktiviert. Sie unterscheiden sich dagegen, wie aus der folgenden Tabelle hervorgeht, in ihrem Verhalten gegen Mangan, Eisen und Selenit. Dieser Befund spricht ebenfalls für eine Verschiedenheit der beiden Enzyme.

Das Ergebnis bei Vergleichung der katheptischen Proteinasen der Leukocyten, Leber und Niere des Meerschweinchens ist in Tab. 2 enthalten. Wie ersichtlich, verhalten sich diese Enzyme durchweg gleichsinnig, mit Ausnahme des Selenits, das auf das Leukocytenkathepsin leicht fördernd, auf die Organkathepsine schwach hemmend einwirkt.

TABELLE 2

Aktivität der katheptischen Proteinasen der Leukocyten, Leber und Niere des Meerschweinchens gegen das Eigeneiweiß unter dem Einfluß von Schwermetallen und Selenit

Aktivität der unbeeinflußten Enzyme = 100%

mg/ccm	Kupfer $7 \cdot 10^{-2}$	Zink $8 \cdot 10^{-2}$	Quecksilber $14 \cdot 10^{-2}$	Mangan $11 \cdot 10^{-2}$	Eisen $7 \cdot 10^{-3}$	Selenit $25 \cdot 10^{-3}$
Kathepsin der						
Leukocyten	86 %	109%	126%	119%	114%	126 %
Leber	77,6%	100%	120%	115%	110%	73,6%
Nieren	60,4%	103%	109%	110%	96%	80 %

EXPERIMENTELLER TEIL

Die im Allgemeinen Teil angeführten Befunde werden im folgenden der Raumbeschränkung wegen nur mit wenigen, typischen Versuchsprotokollen belegt. Bezüglich der Grundlagen der Nephelometrie und der Handhabung des hier benutzten Kleinmannschen Nephelometers sei auf die zusammenfassenden Darstellungen von Kleinmann[17]) verwiesen. Die nephelometrische Analyse proteolytischer Vorgänge an verschiedenen Substraten und mit verschiedenen Enzymen ist in einer Reihe von Arbeiten von Rona und Kleinmann[18]), Kleinmann[19]), Kleinmann und Stern[7]) und Stern[13, 14]) eingehend beschrieben.

Gewinnung der Leukocyten und Herstellung der Extrakte

Gemäß der Hamburger-De Haanschen[12]) Vorschrift wurde bei der Gewinnung der Exsudate derart verfahren, daß mehrere ausgewachsene Meerschweinchen am Vorabend des Versuchstages durch Injection steriler physiologischer Kochsalzlösung (0,9%ig) in die Bauchhöhle in einer Menge von etwa 30 ccm vorbereitet wurden. Am folgenden Morgen wurden weitere 50 ccm der NaCl-Lösung injiziert und nach 4–5 Stunden das gebildete Exsudat mit Hilfe einer Troikartkanüle entleert. War nicht genügend Flüssigkeit vorhanden, so wurde mit NaCl-Lösung nachgespült. Das ausfließende Exsudat wurde in einer 0,6% Natriumcitrat enthaltenden physiologischen Kochsalzlösung aufgefangen und sogleich kurz zentrifugiert. Der Rückstand wurde 2 mal mit Ringerlösung gewaschen, feucht gewogen und nach Entnahme einer kleinen Probe für den Kontrollabstrich in etwa der 25fachen Gewichtsmenge 87%igen Glycerins unter Zusatz von 1 Tropfen n-Essigsäure pro 5 ccm Glycerin verteilt. Die Suspension wurde über Nacht im Thermostaten bei 36–37° belassen und sodann durch ein Faltenfilter filtriert. Das Filtrat diente zu den Spaltungsversuchen und wurde im Kühlschrank aufbewahrt.

Die zu den Vergleichsversuchen dienenden Organextrakte (Leber- und Nierenauszug) des Meerschweinchens wurden nach der von Waldschmidt-Leitz und Deutsch[11]) für die Milz gegebenen Vorschrift hergestellt: Die sogleich nach dem Tode entnommenen Organe mehrerer Tiere wurden im Mörser zerkleinert, mit der doppelten Gewichtsmenge 87%igen Glycerins 4 Stunden bei 37° extrahiert und sodann der nach 24 Stunden durch gewöhnliche Faltenfilter hindurchgegangene Anteil als Auszug verwendet.

In einem Falle wurde auch die frische Suspension von Leukocyten in 87%igem Glycerin als Enzymlösung benutzt.

Von 4 Tieren wurden im Durchschnitt jedesmal 200 mg feuchte Leukocyten gewonnen. Die Vorbereitung mittels Kochsalzinfusion am Vorabend wurde nur beim ersten Versuche vorgenommen. Weiterhin wurde dann nur eine Injektion 3–5 Stunden vor der Exsudatentnahme gemacht.

Die Ausstriche zeigten, daß im Exsudat überwiegend pseudoeosinophile (neutrophile) polynucleäre Leukocyten neben meist großen Mononucleären und vereinzelten eosinophilen Leukocyten vorhanden waren. Meist war auch eine Anzahl roter Blutzellen sichtbar.

Von insgesamt zwanzig verwendeten Tieren wurden im Laufe der Versuche zusammen etwa 5 g feuchte Leukocyten gewonnen, ohne daß auch nur ein einziges Tier erkrankte oder starb.

I. Beispiel: Aktivitäts-p_H-Kurve eines Leukocytenextraktes gegen Serumalbumin[18, 7]) (Rind) als Substrat:

Spaltungsansätze: In diesem wie in allen weiteren Versuchen wurde als Puffer das kürzlich von Michaelis[20]) angegebene Veronal-Acetat-Salzsäuresystem ver wendet, das unter Variation nur einer Komponente (der HCl) erlaubt, das gesamte in Frage kommende p_H-Gebiet zu bedecken. 2,5 ccm Albuminlösung + 2,5 ccm m/7-Veronal-Acetatlösung (9,714 g Natriumacetat + 14,714 g Veronalnatrium, gelöst in 500 ccm Aq. dest.) + n ccm n/10-HCl + Aq. ad 14 ccm. Die Albuminlösung aus käuflichem Rinderserumalbumin in einer Konzentration von etwa 0,5% verwendet.

Nach Vermischen 5 ccm entnommen, 4,5 ccm als Nullabnahme aufbewahrt, die restlichen 0,5 ccm verworfen. Zu den restlichen 9 ccm der Mischung wurde 1 ccm des mit Aq. doppelt verdünnten Leukocytenextraktes M.L. 4, sowie 4 Tropfen Toluol gegeben. Nach 46stündigem Verweilen der Ansätze und der Nullabnahmen im Thermostaten bei 37° wurden aus den Ansätzen je 5 ccm entnommen, zu den Nullentnahmen nunmehr 0,5 ccm der Fermentlösung pipettiert und zu allen Proben je 10 ccm 12,5%iger Salzsäure + 7 ccm einer 20%igen Lösung von Sulfosalicylsäure gegeben. Analyse im Nephelometer von Kleinmann nach 5 Minuten. Im Rest der Spaltmischungen wurde die Wasserstoffionenkonzentration elektrometrisch gemessen.

Nr.	im Ansatz enthalten	p_H	Aufspaltung
1	7,5 ccm 0,1 n-HCl	2,35	10,1%
2	6,8 ccm „	2,79	13,8%
3	5,0 ccm „	**4,36**	**14,8%**
4	4,0 ccm „	4,86	13 %
5	3,0 ccm „	6	10 %
6	1,2 ccm „	7,8	7 %
7	2,5 ccm 0,01 n-NaOH	9,34	2 %

II. Beispiel: Aktivitäts-p_H-Kurve eines Leukocytenextraktes gegen Casein[18, 7]) als Substrat:

Anordnung: 3,65 ccm einer 0,25%igen Caseinlösung (enthaltend 5 g Casein „Hammarsten“ + 12 ccm n-NaOH in 2000 ccm) + 2,5 ccm m/7-Veronal-Acetatlösung + n ccm HCl + Aq. ad 14 ccm·5 ccm für Nullabnahme von 4,5 ccm entnommen. Zu den restlichen 9 ccm 1 ccm des aufs Doppelte mit Aq. verdünnten Leukocytenextraktes M.L.4, sowie etwas Toluol gegeben. 19 Stunden bei 37° gehalten, ebenso die Nullabnahmen. Zur Analyse 5 ccm entnommen, zu den Nullabnahmen ebenfalls 0,5 ccm der Enzymlösung gegeben, das in einigen Ansätzen ausgeflockte Substrat durch Zugabe der unten vermerkten Laugenmenge in Lösung gebracht, das Volumen mit Aq. überall auf 10 ccm ergänzt, ferner 5 ccm eines m/15-Phosphatpuffers von p_H 8 sowie 7,5 ccm einer gesättigten Chinidinhydrochloridlösung zugegeben. Nach etwa 15 Minuten nephelometriert.

Nr.	in dem Ansatz	Aussehen	p_H	zur Analyse	Aufspaltung
1	7,5 ccm n/10-HCl	klar	2,25	2,2 ccm 0,1 n-NaOH	2,4%
2	6,8 ccm „	leicht trübe	2,94	1,5 ccm „	11,2%
3	5,0 ccm „	geflockt	4,33	1,2 ccm „	15,6%
4	4,0 ccm „	„	4,91	1,0 ccm „	24,5%
5	3,0 ccm „	schwach getr.	6,26	—	30,1%
6	1,2 ccm „	klar	8,04	—	23 %
7	2,5 ccm 0,01 n-NaOH	klar	9,3	—	0 %

III. Beispiel: Aktivitäts-p_H-Kurve eines Leukocytenauszuges gegen Eigeneiweiß13) als Substrat:

Anordnung: 2,5 ccm m/7-Veronal-Acetatlösung + n ccm HCl + Aq. ad 14 ccm. 5 ccm entnommen für Nullabnahme von 4,5 ccm. Zu den restlichen 9 ccm 1 ccm des unverdünnten Leukocytenextraktes M.L. 8 gegeben und ohne Toluolzusatz 5 Stunden bei 36° gehalten. Sodann 5 ccm zur Analyse entnommen, zu den Nullabnahmen je 0,5 ccm der Fermentlösung gegeben und zu allen Proben 11 ccm Aq. + 4 ccm der 20%igen Sulfosalicylsäurelösung pipettiert. Es gelangt bei dieser Anordnung allein der Abbau des in den Extrakten enthaltenen Eigenproteins der Leukocyten zur Beobachtung.

Im Restvolumen der Ansätze wurde p_H elektrometrisch gemessen. Sämtliche Ansätze waren klar geblieben.

Nr.	im Ansatz	p_H	Aufspaltung
1	1,2 ccm n-HCl	1,65	0 %
2	7,5 ccm 0,1 n-HCl	2,25	0 %
3	6,2 ccm „	3,5	32,9%
4	4,5 ccm „	4,6	18,4%
5	3,0 ccm „	5,66	25,4%
6	1,2 ccm „	8,07	31,8%
7	1 ccm 0,01 n-NaOH	8,8	20 %

IV. Beispiel: Aktivitäts-p_H-Kurve eines Leukocytenauszuges gegen Edestin14) als Substrat:

Anordnung: 2,5 ccm m/7-Veronal-Acetatlösung + n ccm HCl + 6 ccm einer 0,1%igen Edestinlösung (erhalten durch Lösen von 1 g Edestin in 1000 ccm einer 0,03 n-HCl in der Wärme und Filtrieren) + Aq. ad 14 ccm. Entnommen 5 ccm für Nullabnahme von 4,5 ccm. Zu den restlichen 9 ccm 1 ccm des doppelt verdünnten Leukocytenextraktes M.L. 7 gegeben. Unter Zugabe von einigen Tropfen Toluol 44 Stunden bei 37° belassen. Nunmehr 5 ccm zur Analyse entnommen, zu den Nullabnahmen je 0,5 ccm der Enzymlösung gegeben, das ausgeflockte Edestin durch Zugabe von 0,1 n-HCl gelöst, das Volumen überall mit Aq. auf 18 ccm gebracht und 4 ccm der 20%igen Sulfosalicylsäurelösung zur Erzeugung der Trübung zugefügt. Innerhalb der ersten 10 Minuten nach Reagenzzugabe nephelometriert. Im Rest der Ansätze elektrometrisch p_H bestimmt.

Nr.	im Ansatz	Aussehen	zur Analyse	p_H	Aufspaltung
1	5,5 ccm 0,1 n-HCl	klar	—	2,14	3 %
2	4,0 ccm „	„	—	3,70	31,5%
3	2,0 ccm „	trübe	2,5 ccm 0,1 n-HCl	4,84	20 %
4	1,0 ccm „	geflockt	2,5 ccm „	5,68	48,1%
5	—	„	2,5 ccm „	7,34	60 %
6	1,0 ccm 0,1 n-NaOH	klar	2,5 ccm „	7,92	70,6%
7	2,0 ccm „	„	2,5 ccm „	8,54	31,5%

V. Beispiel: Herstellung und Reinigung einer Kinaselösung aus Schweinedünndarm[16]) und Prüfung ihrer Wirksamkeit auf Meerschweinchen-Pankreastryptase:

Darmschleimhaut-Trockenpulver: Es wurde genau nach der von Waldschmidt-Leitz[16]) gegebenen Vorschrift verfahren:

Aus 100 m des obersten Dünndarmabschnittes ebensovieler Schweine wurden durch Behandlung der mit Glasscherben abgekratzten Schleimhaut mit Aceton und Äther 45 g Trockenpräparat gewonnen. Daraus wurden durch Mahlen in einer Pulvermühle und Sieben durch ein feinmaschiges Drahtsieb etwa 30 g feines Pulver gewonnen.

Kinaselösung Nr. 2: 1 g des Pulvers wurde mit 50 ccm 87%igen Glycerins angeschüttelt und 48 Stunden bei 30° belassen. Das Glycerin wurde vom Rückstand auf der Zentrifuge abgetrennt, mit 25 ccm etwa 20%igen Glycerins nachgewaschen und der Rückstand 3 Stunden bei 30° mit 50 ccm 0,05 n-NH_3 auf der Schüttelmaschine extrahiert. Die durch Zentrifugieren erhaltene kinasehaltige Restlösung wurde 2 Stunden lang mit einem Luftstrom von etwa 37° behandelt, bis sie neutral reagierte. Der auf Zusatz von 2,5 ccm n-Essigsäure entstandene Niederschlag wurde durch Zentrifugieren abgetrennt, die Restlösung mit n-NaOH gegen Lackmus neutralisiert. Am nächsten Tag wurden 5 ccm einer 1%igen Sublimatlösung zugefügt, von dem Niederschlag abzentrifugiert und zur Entfernung des überschüssigen Quecksilbers 30 Minuten H_2S eingeleitet. Nach Abfiltrieren vom HgS wurde zur Vertreibung des Schwefelwasserstoffs im warmen Luftstrom bis auf etwa 10 ccm Volumen eingedunstet und mittels 5 ccm 87%igen Glycerins und Aq. auf 25 ccm aufgefüllt. Aufbewahrung im Kühlschrank.

Prüfung der Wirksamkeit an Meerschweinchen-Pankreastryptase

Herstellung des Meerschweinchen-Pankreasextraktes

Von sechs mittels Äthernarkose getöteten Meerschweinchen wurden insgesamt 4 g frische Pankreasdrüse erhalten. Diese wurde zunächst 16 Stunden lang bei 37° mit 87%igem Glycerin extrahiert (20 ccm). Dann wurden weitere 30 ccm Glycerin und 0,8 ccm Eisessig zugegeben und 6 Stunden bei 30° auf der Schüttelmaschine extrahiert. Durch Zentrifugieren und Filtrieren etwa 40 ccm Pankreasextrakt (P) erhalten.

Prüfung: Es wurden folgende Gemische mit Hilfe dieses Pankreasextraktes und der Kinaselösung bereitet und etwa 60 Minuten bei 37° zum Vollzug der Tryptaseaktivierung inkubiert:

Trypsin allein	Kinase allein	Trypsin-Kinase
2 ccm Pankreasextrakt (P) 1:20 verdünnt + 0,25 ccm H_2O	2 ccm H_2O + 0,25 ccm der Kinaselösung 2.	2 ccm Pankreasextrakt (P) 1:20 verdünnt + 0,25 ccm der Kinaselösung 2.
+ 1,75 ccm m/15-Phosphatpuffer p_H 7,4.	dito	dito

Gesamtvolumen jeder Mischung 4 ccm.

Spaltungsansätze: 5 ccm m/7-Veronal-Acetatlösung + 4 ccm 0,1 n-NaOH + 12 ccm der 0,1%igen Edestinlösung + Aq. ad 28 ccm. 10 ccm für 2 Nullabnahmen à 4,5 entnommen. Zu den restlichen 18 ccm nach Vorwärmen im Thermostaten von 36° je 2 ccm der Inkubationsgemische gegeben; p_H etwa 8,5, Mischungen leicht getrübt, keine Flockung.

Analyse: Aus den Spaltungsgemischen nach 10, 20 und 30 Minuten je 5 ccm entnommen und zur Unterbrechung der Spaltung in 2,5 ccm 0,1 n-HCl einfließen lassen. Auch zu den Nullabnahmen die gleiche Säuremenge sowie 0,5 des betreffenden Inkubationsgemisches gegeben. Alle Proben mit Aq. auf 18 ccm aufgefüllt und mittels 4 ccm der 20%igen Sulfosalicylsäurelösung die Trübung erzeugt, welche im Anschluß daran im Kleinmannschen Nephelometer analysiert wird.

Ergebnis der nephelometrischen Messung:

Spaltzeit	10 Minuten	20 Minuten	30 Minuten
Trypsin allein:	31 %	51,9%	60,5%
Kinase allein:	7 %	5,7%	8,3%
Trypsin-Kinase:	54,8%	75 %	83,9%
Aktivierung:	16,8%	17,4%	15,1%

VI. Beispiel: Negativer Versuch, die Tryptase der Meerschweinchenleukocyten durch die Schweinekinaselösung Nr. 2 gegen Edestin zu aktivieren:

Angewendet den Leukocytenglycerinauszug M.L. 10:

Inkubationsgemische

Trypsin allein	Kinase allein	Trypsin-Kinase
2 ccm Leukocytenextrakt M.L. 10, unverdünnt	2 ccm H_2O	2 ccm Leukocytenextrakt M.L. 10, unverdünnt
+ 0,25 ccm H_2O	+ 0,25 ccm Kinaselösung Nr. 2.	+ 0,25 ccm Kinaselösung Nr. 2.
+ 1,75 ccm m/15-Phosphatpuffer, p_H 7,4.	dito	dito

Volumen jeder Mischung 4 ccm. 35 Minuten bei 37° inkubiert.

Spaltansätze: 5 ccm m/7-Veronal-Acetatlösung + 2 ccm 0,1 n-NaOH + 12 ccm 0,1%ige Edestinlösung + Aq. ad 28 ccm Volumen. 10 ccm für 2 Nullabnahmen à 4,5 entnommen. Zu den restlichen 18 ccm nach Vorwärmen im Thermostaten bei 36° 2 ccm eines der Inkubationsgemische gegeben. p_H etwa 8.

Analyse: Nach 2 und nach 20 Stunden 5 ccm aus den Spaltungsansätzen entnommen, Edestintrübung durch Zusatz von 2 ccm 0,1 n-HCl beseitigt. Zu den Nullabnahmen ebenfalls 2 ccm 0,1 n-HCl sowie 0,5 des betreffenden Inkubationsgemisches zugefügt. Volumen aller Proben mit Aq. auf 18 ccm ergänzt und Trübungsreaktion durch Zugabe von 4 ccm der 20%igen Sulfosalicylsäurelösung erzeugt. Im Anschluß daran nephelometriert.

Ergebnis:

Spaltzeit	2 Stunden	20 Stunden
Trypsin allein	22,5%	52,4% Spaltung
Kinase allein	5,7%	
Trypsin-Kinase	25,4%	54,8% Spaltung

Keine Aktivierung durch die Kinase!

VII. Beispiel: Negativer Versuch der Zerlegung der Leukocytentryptase mittels Aluminiumhydroxyd in Kinase und einen mittels Kinase aktivierbaren Rest[16]):

Anordnung: 5 ccm des Leukocytenauszuges M.L. 10 mit 0,06 ccm n-Essigsäure + 1,5 ccm einer Aluminiumhydroxydsuspension A [nach Willstätter und Racke[21])] versetzt. Zentrifugiert und zur Restlösung weitere 1,5 ccm der Aluminiumsuspension gegeben. Die durch Zentrifugieren erhaltene Adsorptionsrestlösung wurde zu den folgenden Inkubationsgemischen verwendet:

Trypsin allein	Kinase allein	Trypsin-Kinase
3 ccm der Restlösung 0,25 ccm H_2O	3 ccm H_2O 0,25 ccm Kinaselösung Nr. 2.	3 ccm der Restlösung + 0,25 ccm Kinaselösung Nr. 2.
0,75 ccm m/15-Phosphatpuffer p_H 7,4.	dito	dito

Volumen jeder Mischung 4 ccm. 30 Minuten bei 37° inkubiert. Spaltansätze und Analysengemische dieselben wie im VI. Beispiel.

Ergebnis:

Versuchszeit:	4 Stunden	18 Stunden
Trypsin allein	34,4%	60 %
Kinase allein	6,6%	21 %
Trypsin-Kinase	40,3%	63,4%

Es wurde nicht nur keine Aktivierung des in der Adsorptionsrestlösung verbliebenen Trypsins, sondern im Gegenteil nach 18 Stunden eine Hemmung der in der Kinaselösung enthaltenen Trypsinreste durch die Inhaltsstoffe der Leukocyten beobachtet.

VIII. Beispiel: Verhalten der Meerschweinchenleukocytentryptase gegen Kupfer-, Zink-, Quecksilber-, Mangan-, Eisen- und Selenitionen (Substrat: Eigeneiweiß).[13])

Anordnung: 2,5 ccm m/7-Veronal-Acetatlösung + 1,2 ccm 0,1 n-HCl + Salzlösung + Aq. ad 14 ccm. Entnahme von 5 ccm für Nullabnahme von 4,5 ccm. Zum Rest von 9 ccm wird 1 ccm des unverdünnten Leukocytenextraktes M.L. 15 gegeben und das Gemisch 5 Stunden bei 37° gehalten.

Analyse: Aus jedem Ansatz werden 5 ccm entnommen, zu den Nullabnahmen je 0,5 ccm der Enzymlösung zugesetzt und das Volumen aller Proben mit Aq. auf 16 ccm gebracht. Durch Zusatz von 4 ccm einer 20%igen Sulfosalicylsäurelösung wird eine Trübung erzeugt, die sogleich im Nephelometer ausgewertet wird.

Nr.	im Ansatz enthalten	Aufspaltung
1	ohne Zusatz	23 %
2	0,3 ccm einer 1%igen $CuSO_4$-Lösung (= $7 \cdot 10^{-2}$ mg Cu pro ccm)	4,8%
3	0,3 ccm einer 1%igen $ZnSO_4$-Lösung (= $8 \cdot 10^{-2}$ mg Zn pro ccm)	0 %
4	0,3 ccm einer 1%igen $HgCl_2$-Lösung (= $14 \cdot 10^{-2}$ mg Hg pro ccm)	38,5%
5	0,3 ccm einer m/10-$MnSO_4$-Lösung (= $11 \cdot 10^{-2}$ mg Mg pro ccm)	44,9%
6	1,2 ccm einer 0,5%igen Na_2SeO_3-Lösung (= $25 \cdot 10^{-2}$ mg SeO_3 pro ccm)	38,5%
7	0,3 ccm einer 1%igen $FeSO_4$-Lösung (= $7 \cdot 10^{-2}$ mg Fe pro ccm)	21,6%

ZUSAMMENFASSUNG

1. Die Frage des Vorkommens einer echten tryptischen Proteinase in farblosen Blutzellen wurde an einem Versuchsobjekt nachgeprüft, in dem nach den Angaben der Literatur am reichlichsten ein solches Enzym zu erwarten war: An sterilen Exsudatleukocyten des Meerschweinchens. Diese bestehen überwiegend aus myeloischen (polynucleären, neutrophilen) Leukocyten.

2. Unter Heranziehung von nativen Proteinen (Eigeneiweiß und Edestin) als Substrat und Benutzung der nephelometrischen Bestimmungsform, die Störungen durch ereptische Enzyme ausschließt, konnte in den Leukocytenextrakten neben der ubiquitären katheptischen Proteinase eine echte, natives Eiweiß optimal bei p_H 8 angreifende, tryptische Proteinase nachgewiesen werden.

3. Diese Tryptase war in den anderen untersuchten Organen des Meerschweinchens (Leber, Niere) unter den Versuchsbedingungen nicht nachweisbar. In diesen wurde vielmehr nur die im schwach sauren Gebiet optimal wirkende katheptische Proteinase gefunden.

4. Die leukocytäre Tryptase konnte weder durch arteigene noch durch artfremde Kinase in ihrer Wirksamkeit auf Edestin aktiviert werden. Auch eine Zerlegung des tryptischen Komplexes in inaktive Tryptase und Kinase mittels Aluminiumhydroxyd wurde nicht erreicht.

5. Die Tryptasen der Leukocyten und der Pankreasdrüse unterscheiden sich weiterhin durch ihr Verhalten gegen kleine Mengen von Mangan-, Eisen- und Selenitionen. Gegen Kupfer-, Zink- und Quecksilberionen verhalten sie sich gleichsinnig.

6. Die katheptischen Proteinasen der Leukocyten, Leber und Niere

verhalten sich gegen alle zugesetzten Schwermetallionen gleichsinnig, gegen Selenitionen verschiedenartig.

7. Aus den Versuchen kann geschlossen werden, daß die Tryptase der Leukocyten und des Pankreas trotz gleichen p_H-Optimums und gleicher Spezifität wahrscheinlich zwei selbständige Enzyme verschiedener Herkunft darstellen.

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20. L. Michaelis, Biochem. Z. **234**, 139 (1931).
21. R. Willstätter u. Racke, Liebigs Ann. **425**, 1 (1920/21).

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A POTENTIOMETRIC STUDY OF WURSTER'S RED AND BLUE

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In a rather large number of cases there have been found compounds intermediary, with respect to the level of oxidation, between a compound of the quinoid type and one of the hydroquinoid type. In a previous paper[1] it could be shown by a potentiometric method for three organic dyestuffs of the oxyphenazine series, that this intermediary compound is a molecule of the same size as either the holoquinoid form or the hydroquinoid form and differs from the hydroquinoid only by one oxidation equivalent (or hydrogen atom or electron). It must be considered as a radical-like compound with an odd number of electrons. Those three dyes offered a particularly favorable opportunity for the proof of such a statement because the intermediary compounds were very stable and permitted a thorough potentiometric study of the system, there being no drift in the potentials in time. The conclusions were based on observations of systems in a true thermodynamical equilibrium and therefore were scarcely liable to a misinterpretation.

This observation suggested the idea that other examples of half-reduced quinoid substances might be accessible to a similar potentiometric analysis. The difficulty in most of these cases is the fact that these intermediary compounds are as a rule not very stable in aqueous solution but undergo irreversible reactions and so cause a drift of the potentials. An attempt to utilize the potentiometric method for such a purpose was made by Clark, Cohen and Gibbs,[2] who studied the so-called meriquinones formed from aromatic diamines. They took for granted the current idea that these intermediary compounds are meriquinones according to Willstätter's[3] definition as being molecular compounds of the holoquinoid and the hydroquinoid form.

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[1] Michaelis, *J. Biol. Chem.*, in press, July (1931).

[2] W. Mansfield Clark, Barnett Cohen and H. D. Gibbs, *Public Health Repts.*, Suppl. No. 54, 1 (1926).

For some years an increasing number of papers has been published advocating the formula of a free radical in such cases in which formerly double molecules were supposed to exist. In some of these cases an equilibrium between the free radical and its polymeric saturated form was assumed. This holds for instance for Gomberg's triphenylmethyl and for Wieland's biphenylnitride. In other cases the existence of the free radical without its polymerized form was assumed. This holds, for instance, for the case of the aromatic diamines, the subject also of this paper. Weitz and Fischer[4] showed by determination of the boiling point in solutions of such compounds that only the formula of a free radical is compatible with the data observed. A thorough compilation of the whole subject has been published by Weitz[5] in which many other examples are enumerated. Yet never has a potentiometric method been applied for the proof of such a contention. Such a method may not be applicable for every case, but in those cases in which it can be used this method is much superior to any other. All those assumptions with respect to incomplete dissociation which have to be considered in order to correct the boiling or freezing point data for electrolytes do not appear in the potentiometric method. This method whenever it is available may be considered as the best method for the determination of a molecular weight for an ionic compound.

The potentiometric analysis presented in this paper was suggested by, and is based on, a set of half-quantitative, preliminary observations which will first be described. These observations hold for asymmetric dimethyl-*p*-phenylenediamine and tetramethyl-*p*-phenylenediamine. There is no doubt that the method can be extended to other substances of this type but as we have to deal with very labile systems, each of these cases should be studied separately and may require long experimentation before a definite understanding is attained. Therefore this study will be restricted to the two substances mentioned.

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[3] Willstätter and Mayer, *Ber.*, 37, 1494 (1904); Willstätter and Pfannenstiel, *ibid.*, 37, 4605 (1905); Willstätter and Piccard, *ibid.*, 41, 1458 (1908).

[4] Weitz and Fischer, *Ber.*, 59, 432 (1926).

[5] Weitz, *Z. Elektrochem.*, 34, 538 (1928).

When either of the two mentioned diamines is titrated with an oxidant such as bromine or chlorine, a deep color is developed, called Wurster's red in the case of the dimethyl compound and Wurster's blue for the tetramethyl one. When the titration is performed rather rapidly and farther extended, the color disappears again. When, after reaching the point of color discharge, the solution is allowed to stand, the color gradually turns back, though not to its maximum intensity, at a rate largely dependent on concentration and *P*H. The spectroscopic analysis shows that it is the same substance which reappears, as recognizable from the characteristic double band, which differs in Wurster's red and blue only by the spectral location of the two bands. It should not be asserted, however, that the return of the color is due only to the reappearance of Wurster's red or blue. According to the conditions other dyes may or may not be developed besides.

The interpretation of this phenomenon is this. Wurster's red or blue differs from the mother substances, the respective diamines, only by one oxidation equivalent (or hydrogen atom, or electron) as Willstätter and his co-workers[8] have shown long ago. The disappearance of the color in the second step of titration indicates that the dye is oxidized to a higher level which has practically no, or very little, color. One might expect this substance to be the second step of oxidation, analogous to diiminoquinone, and very likely it is. But these substances are extremely labile, as Willstätter has shown; in aqueous solution they spontaneously undergo irreversible changes and are converted into substances to which we must attribute a great inclination to still further oxidation. So these secondary substances will reduce those molecules of the diiminoquinone still present in the solution, to Wurster's red (or blue) over again. From these rough observations we may expect that the second half of the titration is characterized by a very labile system which at no point of the titration is in any chemical equilibrium within reasonable time. So we may expect that even in the best case only in the first half of the titration steady potentials might be established.

This is exactly what happens. When during the titration the potential at a blank platinum electrode is read, there are sufficiently constant and reproducible potentials in the first half of the titration. This holds at least for a certain range of *P*H. The most suitable *P*H for constant potentials proved to be 4.6 for both substances. In the second part of the titration no constant potentials can be obtained under any condition. The potentials always drift back to the negative side as though the oxidized form (the diiminoquinone) would spontaneously and irreversibly disappear in time. When the second half of the titration is performed rather hastily, the potential will stay within a small, not very well reproducible, range and the end of the titration will sometimes be indicated by a sudden though often not very big jump, which, however, drifts back at a rate depending upon the conditions, such as the time required for the whole titration, the number of steps in which the whole titration had been subdivided and the pause between the successive steps. The color vanishes simultaneously with this potential jump and, on standing, reappears, simultaneously with the back drift. If it happens that no jump of the potential appears, no complete discharge of color will be observed either.

Thus the end-point of the first step of titration can, with a fair approximation, be recognized by the fact that the potentials, which were constant up to this point, begin to drift; and the end of the second step can be recognized, provided the titration in the second step is rapidly performed, by the point of coincidence of potential jump and transient discharge of color, if there is such a point at all. The amount of oxidant used for each of the two steps turns out to be the same, within the limits of error in such a labile system.

A theoretical analysis of the titration curve is, therefore, not difficult for the first half of the titration, but impossible at the present time for the second half. When the titration curve of the first step is analyzed, it turns out to be the curve for a regular reversible oxidation-reduction system, and the difference between the reduced and oxidized form turns out to be only one electron. It is very important to add that the mid-point potentials E_0 of this step of oxidation for different experiments, at the same *P*H, coincide, independently of the initial concentration of the diamine.[6] Hereby it is proved that Wurster's red or Wurster's blue differs from its particular diamine only by one electron, without any change in molecular size and that it is not a meriquinone according to Willstätter's definition, namely, a compound of one or several molecules of the holoquinoid form with one or several molecules of the completely reduced form. These two dyes, Wurster's red and blue, therefore are of the type of a half-reduced quinone, or semiquinone, a molecule with an odd number of electrons. We may also say that one of the two nitrogen atoms is bivalent as in Wieland's biphenylnitride. In contrast, however, to these biphenylnitrides, or to Gomberg's triphenylmethyl, the unsaturated radical-like compound does not seem to undergo to any measurable extent a polymerization to a double molecule in which the state of unsaturation is abolished. This radical-like compound in aqueous solution of a *P*H around 4.6, is, though not strictly stable, yet a fairly stable substance, provided there is no excess of oxidant which can oxidize it partly to a higher level. If this is the case, the whole system becomes unstable, and the radical-like compound will partake in the chemical processes which go on with greater or less rapidity. The radicals do not combine with each other to form double molecules or with the original diamine to form quinhydrones, but they undergo condensation or other reactions with the other molecules in the solution, especially with the very unstable substances which are on the oxidation level of diiminoquinones.

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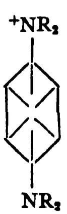
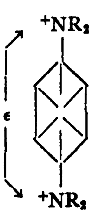
[6] As to significance of the independence of E_0 of the initial concentration, see Ref. 1.

In formulating radicals of the described kind one may accept a point of view proposed already by Weitz, which is best presented in this way. In the static Formula I, where R is either a methyl group or hydrogen, it is indicated that one amino group has one electron less than would correspond to an electroneutral state.



I



II

This static model should be replaced by a more dynamic one (Formula II). One electron has a circuit around both N atoms, or the two nitrogen atoms share one electron. This, then, is a chemical bond consisting in one shared electron, instead of the two in the ordinary chemical bond of G. N. Lewis.

EXPERIMENTAL

The purest commercial grades of the hydrochlorides of dimethyl-*p*-phenylenediamine and of tetramethyl-*p*-phenylenediamine (preparations of Eastman) were recrystallized by dissolving in methyl alcohol and precipitating with benzene. A suitable amount of the crystals was dissolved in deaerated water, and a suitable portion added to 15 cc. of acetate buffer of P_H 4.62 (solution 0.1 normal both with respect to sodium acetate and acetic acid). A freshly prepared solution of bromine was used as oxidant, in such a concentration that 1 to 2 cc. was used to the complete titration of the first step. The titration was performed in a constant temperature chamber at 30° in a stream of nitrogen purified over copper at 450°, though this precaution might not have been imperative in working on such relatively positive potential ranges as we have to deal with here. Three blank platinum electrodes were immersed in the solution. Whenever the potential was steady it was observed for all three electrodes, without there ever being a difference of more than 0.1 millivolt. As soon as the potential began to drift, in the progress of titration, the readings had to be hastened and according to the circumstances only one electrode was used for the potential readings. In the diagrams all potentials, constant over a reasonable time within one or two tenths of a millivolt, are marked with plain circles. For all potentials with a drift the direction of the drift is indicated by an arrow. The short arrows indicate a drift of some tenths of a millivolt per minute; the long arrows indicate a drift of several millivolts per minute. The drawn out curves are those calculated according to

$$E = E_0 + 0.0601 \log \frac{X}{A - X}, \text{ volts}$$

where E_0 is a constant, X the amount of bromine solution in cc., and A that amount of bromine solution used up at the end of the first reversible step of the oxidation. The value of A was determined by the following three methods.

1. The first method is only roughly approximate and cannot be applied in every case; it can only be used when the second step of titration is performed within a few seconds without reading the potentials except for the very last part. This method can only be applied

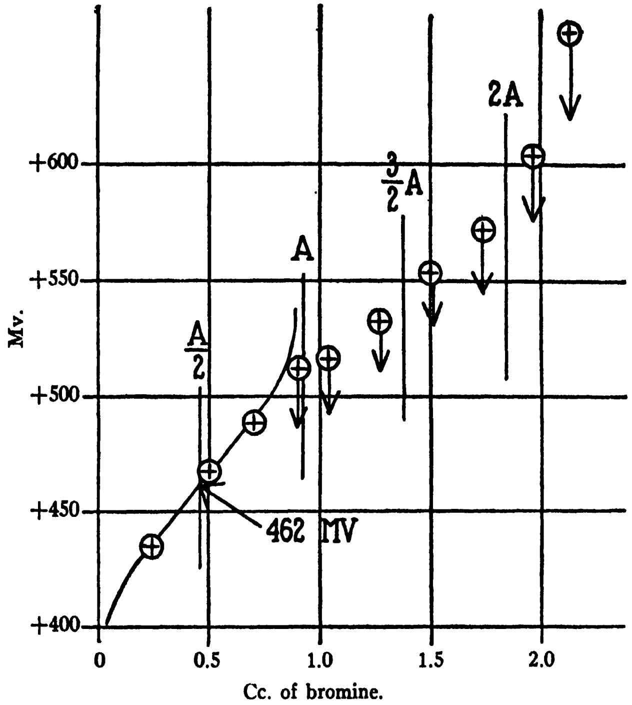


FIG. 1.—2 × 10^{-4} mole of dimethyl-*p*-phenylenediamine hydrochloride dissolved in 20 cc. of acetate buffer of *P*H = 4.62 (a solution 0.1 molar both for sodium acetate and for acetic acid) is titrated at 30°C. with an aqueous solution of bromine. Abscissa, cubic centimeters of bromine. Ordinate, potentials, in millivolts, referred to the normal hydrogen electrode. Arrows signify drift of potential. Potentials without arrow are stable. *A* is the total theoretical amount of bromine used for the complete oxidation to the level of the semiquinone.

when the end of the second step of titration can be recognized by a transient complete discharge of color and a transient jump of the potential. These two phenomena coincide. On account of the unavoidable secondary reactions during the second half of titration even on rapid titration, the observed end-point, if there i all, will always come a little later in the curve than where th ical end-point should lie. If such an end-point can be rec ne may assume that the amount of oxidant at this end-point ximately equal to, or, more precisely speaking, is somewhat greater than, 2 *A*.

2. That point of titration where the previously steady potentials become inclined to a backward drift, can in the first approximation be taken as the value of *A*. This method, also, is an approximative one,

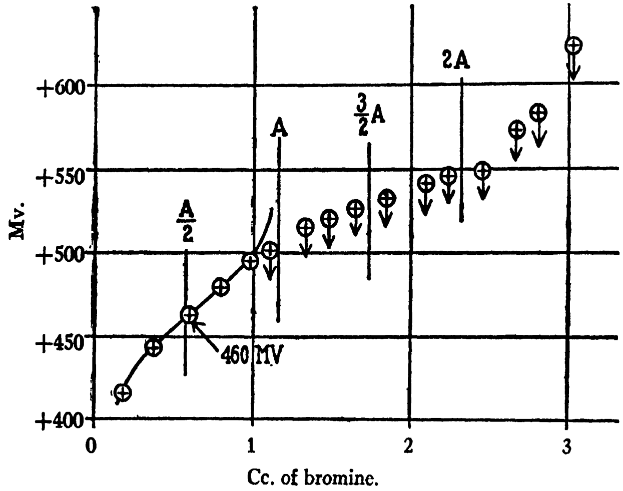


FIG. 2.—4 × 10^{-5} mole of dimethyl-*p*-phenylenediamine hydrochloride dissolved in 20 cc. of acetate buffer titrated with a bromine solution. Designations the same as in Fig. 1.

because a little overlapping of the two steps of titration takes place. The correction of the true value for *A* lies in such a direction that the first drifting potential must occur somewhat before, but never after, the theoretically required amount, *A*, of the oxidant has been added. The result of this method agreed with that of the first within the limits to be expected, namely, that the first method furnished always a somewhat higher value for *A* than the second. The true value of *A* may be estimated as lying between those obtained with the first and those obtained with the second method.

3. After the value of *A* had been evaluated approximately by one

or both of these two methods, a correction was applied in the following way. By a graphical method of trial and error a value of *A* was chosen which furnished the best fitting curve for the first half of the titration where the potentials show no drift. It was postulated that there exists some value of *A* that will furnish an entirely fitting curve, and that this value of *A* should be very close to the one obtained with the two other methods, the deviation lying in the expected direction. This postulate was fulfilled in every case.

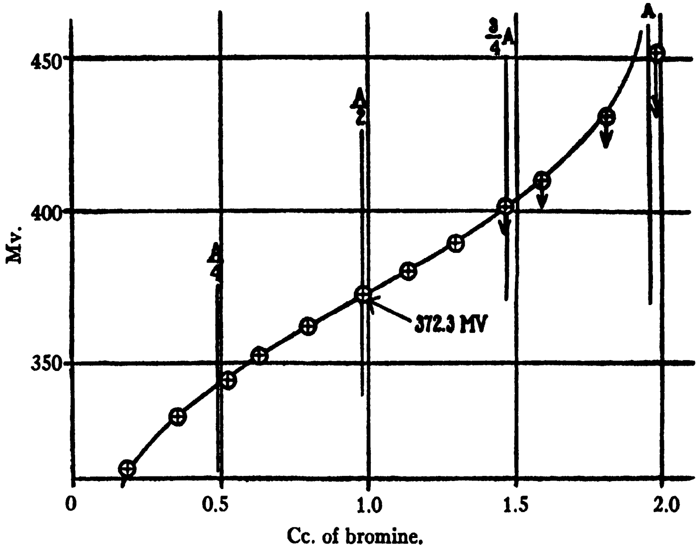


FIG. 3.—1.0 × 10^{-5} mole of tetramethyl-*p*-phenylenediamine hydrochloride dissolved in 15 cc. of acetate buffer and titrated with bromine. Designations as in Fig. 1. The short arrows signify a drift of the potential of about 0.1 millivolt per minute; the long arrow, a drift of several millivolts per minute. Potentials without arrow are perfectly stable.

So the final and definite method is the third one, and the two others were only used to show that the value, *A*, obtained with this method can be shown, in suitable cases, to be compatible with those values which might have been approximately determined by the two others.

In any event, the third method furnished a theoretical curve which, except for the very end of this curve, for understandable reasons, fitted perfectly with the observations, and which furnished E_0 values agreeing satisfactorily for different experiments even on varying the initial concentration in the ratio 1:10. There is, then, every justification for utilizing these curves for an interpretation based on thermodynamical considerations.

There is a slight difference between the two amines with respect to the third method. In the tetramethyl compound the overlapping of the first and second steps of oxidation is somewhat greater than in the dimethyl compound. This is recognizable by the relatively earlier beginning of the drifting part of the curve. This disadvantage is, however, compensated by the fact that the potentials of the tetramethyl compound, before the drifting part of the curve begins, are even more stable than for the dimethyl compound. The tetramethyl compound, during the first, not drifting, part of the curve, can be titrated with the same leisure as any good reversible system; furthermore, even when the drift begins, this drift is so slow (within some tenths of a millivolt per minute) that the uncertainty of the potential value may lie only within one millivolt. Here the drift does not cause so much a time distortion of the curve, but rather shows the beginning of an overlapping and marks that part of the curve beyond which the

TABLE I

1.3×10^{-6} Moles of Tetramethylphenylenediamine Hydrochloride Dissolved in 15 Cc. of Acetate Buffer, PH 4.62, and Titrated with Bromine

E calculated according to the formula $E = 0.1303 + 0.0601 \log \dfrac{X}{1.660 - X}$ volts

X (cc. of Br solution)	Percentage of oxidation, the whole first step of oxidation taken as 100%	Drift of the potential in millivolts per minute (approximately)	E in volts referred to the calomel electrode (add 0.2420 volts to obtain the potential referred to the normal H_2-electrode)	
			Observed	Calcd.
0.280	16.9	0	+0.0882	+0.0887
.465	28.0	0	.1048	.1057
.620	37.3	0	.1160	.1168
.805	48.5	0	.1285	.1287
.990	59.6	0	.1401	.1405
1.154	69.5	0	.1517	.1518
1.320	79.4	0.1	.170	.1658
1.610	98.0	5.0	.201	.1961

potentials can no longer strictly obey the simple formula calculated for a one-step oxidation without overlapping.

These E_0 values must be considered as being strictly indicative of a free energy level of the system, and from the shape of the curve it can be decided with safety which one of the two reactions

$$1 \text{ mole reduced form} \rightleftharpoons 1 \text{ mole oxidized form} + \epsilon \quad (1)$$

(Wurster's red as semiquinone)

$$2 \text{ moles reduced form} \rightleftharpoons 1 \text{ mole oxidized form} + \epsilon \quad (2)$$

(Wurster's red as meriquinone)

(ϵ is one electron) takes place. The first of these two possibilities, then, is found to be the correct one.

Two titration experiments are shown for dimethyl-*p*-phenylenediamine graphically in Figs. 1 and 2. The initial concentrations differ in these two cases in the ratio 1:5, the greater one being 0.01 molar. Two experiments are shown for tetramethylphenylenediamine, one as a graph (Fig. 3) and another in the form of Table I. The initial concentrations in these two experiments differ in the ratio 1:7.5, the greater one being 0.003 molar.

SUMMARY

It is shown by mathematical analysis of the potential curve obtained by oxidative titration with bromine that Wurster's red and Wurster's blue are not molecular compounds of one molecule of a quinoid type with another molecule of a hydroquinoid type, but true semiquinones, *i.e.*, radical-like compounds intermediary between quinone and hydroquinone and of the same molecular size as each of these two. Such a compound must possess an odd number of electrons. One may express the situation by saying that the two nitrogen atoms of the dye are linked by a chemical bond which consists of a single electron instead of a pair.

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NACHTRAG ZU DER ARBEIT: METHÄMOGLOBINBILDUNG UND ATMUNGSSTEIGERUNG DURCH ORGANISCHE FARBSTOFFE[1]

Von L. MICHAELIS

(*Aus den Laboratorien des Rockefeller Institute for Medical Research*)

(Eingegangen am 30. Juni 1931)

Herr *Wolfgang Heubner* macht mich darauf aufmerksam, daß in der im Titel zitierten Arbeit mehrere Arbeiten aus seinem Institut nicht berücksichtigt worden sind.[2] In der Tat sind die in diesen Arbeiten beschriebenen vergleichenden Versuche mit Ferricyankalium und mit Chinon in mancher Beziehung ähnlich denen in unserer Arbeit mit organischen Farbstoffen. Der Grund, weshalb wir gerade Chinon vermieden, obwohl es doch als der einfachste Repräsentant des Typus der organischen Farbstoffe betrachtet werden kann, ist folgender.

Um eine Voraussage über die Menge des entwickelten Sauerstoffgases bei der Umwandlung von Oxyhämoglobin zu Methämoglobin zu machen, muß man wissen, wieviel Sauerstoff der durch die Methämoglobinbildung reduzierte Stoff bei seiner etwa stattfindenden Reoxydation verbraucht. Für Ferricyankalium als Methämoglobinbildner kann diese Frage einwandsfrei beantwortet werden: das durch Reduktion entstandene Ferrocyankalium verbraucht gar keinen Sauerstoff, es wird nicht mit irgendeiner in Betracht kommenden Geschwindigkeit durch O_2 reoxydiert. Für die organischen Farbstoffe ist die Antwort ebenfalls klar: 1 Mol Leukofarbstoff verbraucht $\frac{1}{2}$ Mol O_2 zur Reoxydation. Es ist hierbei belanglos, ob diese Reoxydation wirklich ganz spontan geschieht oder durch Vermittlung eines Katalysators, wie etwa einer Spur eines Kupfersalzes.[3] Ein solcher Katalysator ist immer vorhanden, wenn man ihn nicht durch ganz besondere, kunstreiche Vorsichtsmaßregeln ausschließt. Vielleicht ist bei unserer Versuchsanordnung der Katalysator gar nicht einmal erforderlich, denn *Reids* Versuche beweisen seine Notwendigkeit nur für saure Lösungen, während unsere Versuche bei etwa p_H 7 verlaufen. Es ist ferner belanglos, ob O_2 von dem Leukofarbstoff zu H_2O oder nur zu H_2O_2 reduziert wird, denn H_2O_2 ist in Gegenwart von Blutfarbstoff nicht auf die Dauer existenzfähig.

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[1] *L. Michaelis* u. *K. Salomon*, diese Zeitschr. **234**, 107, 1931.

[2] *W. Heubner*, Arch. f. exper. Pathol. u. Pharm. **72**, 241, 1913; *W. Heubner* u. *H. Rhode*, ebendaselbst **100**, 117, 1924; *Rolf Meier*, ebendaselbst **100**, 127, 1924; **108**, 280, 1925.

[3] *A. Reid*, Ber. **63**, 1920, 1930.

Bei Chinon dagegen sind die Verhältnisse unübersichtlich, wenigstens auf Grund der bisherigen Kenntnisse seiner Eigenschaften. Sein Reduktionsprodukt, Hydrochinon, wird mit genügender Geschwindigkeit nur in stark alkalischer Lösung von O_2 oxydiert. In neutralen Lösungen ist es wenig oder nicht empfindlich gegen O_2, aber die Frage der Katalysatoren spielt hier wohl eine noch wichtigere Rolle und ist noch nicht zur Zufriedenheit geklärt. *Heubner* und seine Mitarbeiter rechnen damit, daß Hydrochinon unempfindlich gegen O_2 ist. Dies wird ja in ihren Versuchen annähernd der Fall gewesen sein, aber es wird eine Komplikation geschaffen, deren Aufklärung nicht zum Problem unserer Arbeit gehörte.

Noch einen zweiten Punkt möchte ich aus *Heubners* Arbeiten zur Erörterung bringen. *Heubner* und *Rhode* beobachteten, daß eine dialysierte Lösung von Methämoglobin beim Durchlüften allmählich wieder zu Oxyhämoglobin wird. Sie deuten dies durch die Annahme eines Gleichgewichtszustandes zwischen Sauerstoff, Hämoglobin, Oxyhämoglobin und Methämoglobin. Diese Annahme ist auf Grund der folgenden thermodynamischen Überlegung als unannehmbar zu erklären.

Hämoglobin wird vollständig zu Methämoglobin oxydiert durch eine Menge von Ferricyankalium, die mit Berücksichtigung der Stöchiometrie dieser Reaktion gerade die äquivalente Menge, oder jedenfalls nicht wesentlich größer als die äquivalente Menge ist. Daraus muß man schließen, daß das Bereich des Oxydations-Reduktionspotentials des Ferri-Ferrocyanidsystems sehr wesentlich positiver ist als das des Methämoglobin-Hämoglobinsystems. Wäre jenes nämlich nur gerade ein wenig positiver (sagen wir um 0,03 Volt), so würde man zur vollständigen Oxydation einen großen Überschuß von Ferricyankalium über die stöchiometrisch verlangte Menge verbrauchen. Nun ist aber das Potentialbereich des Sauerstoffs noch sehr viel positiver als das des Ferricyanidsystems, nämlich um den Betrag von etwa 0,4 Volt bei $p_H = 7$. Dies ist ein gewaltiger Unterschied. Daher ist thermodynamisch zu postulieren, daß Hämoglobin durch die stöchiometrischäquivalente Menge O_2 erst recht restlos zu Methämoglobin oxydiert werden kann. An dieser Betrachtung ändert sich recht wenig, wenn man selbst berücksichtigt, daß in Gegenwart von Luft nur wenig Hämoglobin neben Oxyhämoglobin vorhanden ist. Es ist immerhin mindestens 1°/₀₀ der Menge des Gesamthämoglobins als O_2-freies Hämoglobin vorhanden. Wenn unter diesen Umständen schon eine äquivalente Menge Ferricyankalium das Hämoglobin vollständig zu Methämoglobin oxydieren kann, so muß Sauerstoff dies um so mehr können.

Diese Betrachtung sagt allerdings nichts aus über die Geschwindigkeit, mit der diese Oxydation verläuft, und diese Geschwindigkeit ist in der Tat für Sauerstoff fast unmeßbar klein, wenn nicht ein Katalysator eingreift. Bei Gegenwart eines Katalysators sollte aber die Geschwindigkeit meßbar werden. Wenn wir nun, bei Anwendung gewisser organischer Farbstoffe als Katalysatoren, die Reaktion nicht bis zu dem erwarteten Ende führen konnten, so zeigt das nur, daß der Katalysator infolge irgendwelcher Umstände allmählich aufhört, ein Katalysator zu sein. Der Prozeß kommt zu einem Stillstand, aber nicht zu einem Gleichgewicht, es ist ein Problem der Kinetik, nicht der Thermodynamik.

Nun erfordert noch die Beobachtung von *Heubner* und *Rhode*, daß dialysierte Methämoglobinlösungen beim längeren Durchlüften teilweise zu Oxyhämoglobin regeneriert werden, eine Erklärung. *Heubners* Erklärung, daß ein Gleichgewicht zwischen Methämoglobin und Hämoglobin (und daher auch mit Oxyhämoglobin) bei Gegenwart von molekularem Sauerstoff bestehe, haben wir ja abgelehnt und stehen vor der Aufgabe, sie durch eine andere Erklärung zu ersetzen. Man möchte entweder an bakterielle Wirkungen denken; Bakterien können Methämoglobin zweifellos selbst bei Anwesenheit von Sauerstoff reduzieren. Man könnte auch an Lichtwirkung denken. Im Licht scheinen ja die Gleichgewichte der Blutfarbstoffe (wie auch ihre Reaktionsgeschwindigkeit mit Sauerstoff) wesentlich modifiziert zu werden.[1] *Heubner* glaubt allerdings, beide Faktoren ausgeschlossen zu haben. Und so wird man wohl diese Frage von neuem experimentell in Angriff nehmen müssen, bevor man sich entschließt, wegen eines nicht ganz geklärten Versuchs den zweiten Hauptsatz der Thermodynamik zu stürzen.

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[1] *Hasselbalch*, diese Zeitschr. 19, 435, 1909, und zwar S. 461.

Ich möchte, einer Anregung des Herrn *Heubners* gern folgend, bei dieser Gelegenheit zum Ausdruck bringen, daß wir in der historischen Darstellung der Auffassung des Methämoglobins als einer Ferriverbindung *W. Küster*,[1] *B. v. Reinbold*[2] sowie *W. Heubner* unt Mitarbeiter hätten zitieren sollen. Es ist *Küster*, der Methämoglobin als Ferriverbindung zuerst erkannt hat.

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[1] *W. Küster*, Zeitschr. f. physiol. Chem. 66, 195, 1910, und zwar S. 244.

[2] *B. v. Reinbold*, ebendaselbst 85, 250, 1913.

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COBALT COMPLEXES OF CYSTEINE

By MAXWELL P. SCHUBERT

(From the Laboratories of The Rockefeller Institute for Medical Research)

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The necessity of a more thorough working out of the chemistry of the heavy metal internal complexes of thiol acids arises out of the great interest shown lately in the catalytic oxidation by iron salts of thiol acids to the corresponding disulfide acids. The beginning of such a purely descriptive chemical study was made with cobalt and thioglycolic acid.[1] The study has now been extended and in this paper will be described the preparation and properties of complexes of cobalt and cysteine.

In the case of cobalt and thioglycolic acid only one type of complex could be found, of which several derivatives were prepared in crystalline form. The complex nucleus in these compounds contains two thioglycolic acid radicals for each cobalt atom and is at an oxidation level corresponding to cobalti bis thioglycolate. The situation with cobalt and cysteine was soon found to be more complicated as crystalline complexes were prepared containing cobalt and cysteine in the ratios one to one, one to two and one to three. With cobalt and cysteine the study has also been extended to the isolation of complexes in the reduced state by working in the absence of air. In these the cobalt is in the bivalent condition. Finally, one very striking difference between the cysteine complexes and the thioglycolic acid complexes has been partially cleared up. It has been noticed by Michaelis, but not published,[2] that the olive-brown color of the cobalt cysteine complex made in the presence of air could be changed by the addition of hydrogen peroxide to an intense yellow, much more sensitive than the brown for colorimetric work. There is no similar reaction with the cobalt thioglycolic acid complex.

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[1] L. Michaelis and M. Schubert, This Journal, 52, 4418 (1930).

[2] L. Michaelis and S. Yamaguchi, *J. Biol. Chem.*, 83, 367 (1929).

The Interrelations among the Cobalt Cysteine Complexes.—These are most simply brought out in the accompanying diagram. The simplest of the complexes is I. It is formed by mixing deaërated solutions of cysteine, potassium hydroxide and cobalt chloride in an atmosphere of oxygen-free nitrogen. The *P*H of the resulting solution is probably about 11 to 12. If the *P*H be lower, about 7 to 8, a grass-green crop of needles, II, is obtained. II appears to be the cobalt salt of the same complex nucleus that is contained in I. II is quite insoluble in water, but in dilute potassium hydroxide it undergoes decomposition by precipitating half its cobalt as cobaltous hydroxide while the rest goes into solution as an olive-brown complex. This color is due to the oxidation by air of the complex nucleus in I. If II is decomposed by alkali in an oxygen-free environment, the resulting solution has the green color of I.

Oxidation of the reduced green complex can give rise to at least three different series of complexes. Oxidation with air gives as the principal product III if the *P*H be in the neighborhood of 8–9, while IV is the main product if the solution is more acid, probably about 5. If the *P*H is too high, over 11, it becomes very difficult to isolate any crystalline products at all. Both III and IV are best isolated as free acids. The free acid of IV is insoluble in water and can be obtained in about 85% yields with only a little cystine as by-product. III, on the other hand, can be obtained at best in yields of only about 60% together with a much larger amount of cystine. This fact is in harmony with the idea that in the process of oxidation of complex I, IV is first formed and then by further oxidation two molecules of IV give III and a molecule of cystine. This idea has already been expressed by Kendall and Holst.[3] On the other hand, it has not been found possible, once III and IV are formed, to convert either into the other; for example, heating III with excess cysteine does not give IV and heating IV with alkali or hydrogen peroxide does not produce III. Certainly under the condition of formation of III or IV, IV cannot be converted to III.

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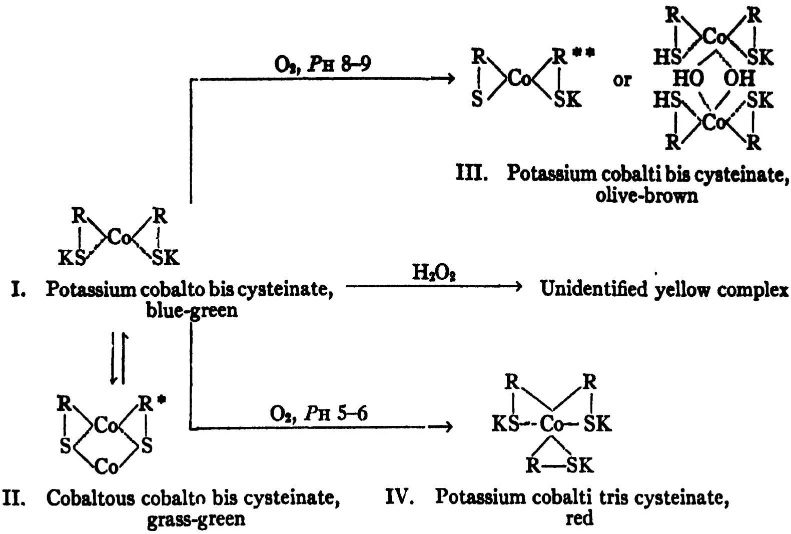
[3] E. C. Kendall and J. E. Holst, *ibid.*, 91, 435 (1931).

R = $OOCCHNH_2CH_2$



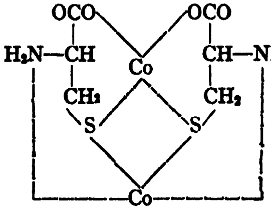
III. Potassium cobalti bis cysteinate, olive-brown

I. Potassium cobalto bis cysteinate, blue-green

Unidentified yellow complex

II. Cobaltous cobalto bis cysteinate, grass-green

IV. Potassium cobalti tris cysteinate, red



* The suggestion that this may be a double internal complex of the following formula will be considered in a later paper.

** As this compound is hydrated, it may be that the normal six coördination places of the trivalent cobalt are completed by the addition of two molecules of water.

Of course the possibility remains that there may be another complex isomeric with IV which acts as the parent substance to III. According to Werner's ideas on the space arrangements of such molecules, IV could exist in two stereoisomeric forms and as a matter of fact the complexes of glycine and alanine[4] with cobalt, which have the same general formula as IV, do exist in two isomeric forms which are supposed to correspond to the possible *cis* and *trans* for [illegible] ut, so far, with cysteine, only one form of IV has been found.

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[4] H. Ley and H. Winkler, *Ber.*, **42**, 3894 (1909); **45**, 372 (1912).

In connection with the alanine complexes just men[illegible] interesting possibility arises. Alanine coördinates with [illegible] through its carboxyl and amino groups. Cysteine has these two groups in the same relative positions and in addition a thiol group. Does cysteine then, coördinate with cobalt through its amino or its thiol group or through both, as well as with its carboxyl group? In other words, does cysteine occupy two or three coördination places on cobalt, and if two, which two of its groups are coördinated?

The Constitution of the Cobalti Bis Cysteinate Complexes.—Two independent lines of evidence agree with a structure of the complex nucleus III which involves coördination of the carboxyl group and the thiol group leaving the amino group of the cysteine free. The first evidence comes from a comparison of the ultraviolet absorption spectra of solutions of the cysteine complex and of the corresponding thioglycolic acid complex, Fig. 1. Since in the case of thioglycolic acid the coördination must take place through the carboxyl and thiol groups, and since the similarity of absorption spectra probably signifies a similarity of structure, it follows that the cysteine complex probably involves coördination of the carboxyl and thiol groups.

Another line of evidence for this comes from a study of the amino group. This method has already been used by Mann[5] in studies on the metal complexes of 1,2,3-triaminopropane to determine how many of these amino groups are coördinated. It is based on the idea that the free electron pair of the nitrogen of an amino group acts as the coördinate link when an amino group coördinates with hydrogen ion or a metal ion. Since there is but one such pair, an amino group cannot coördinate with more than one element at a time. In other words, if the amino group of cysteine is coördinated with the cobalt, it cannot also coördinate with hydrogen ion to form an ammonium salt, a hydrochloride or picrate, for example.

The free acid of III crystallizes in hexagonal plates and complicated patterns of these, and is not very soluble in water but dissolves readily in dilute hydrochloric acid. From concentrated solutions in four to five molar hydrochloric acid a beautifully crystalline crop of olive-brown needles can be obtained. These cannot be washed with water as they revert to the hexagonal crystals. If they are dried over sulfuric acid in a vacuum desiccator, great quantities of fumes of hydrochloric acid pour out when the desiccator is opened. Evidently the hydrogen chloride is held only loosely. The same can be said of picric acid. On treatment of an acid solution containing the complex nucleus of III with picric acid, a crop of clean needle-like crystals separates. These can be recrystallized from water saturated with picric acid but not from water alone, as dissociation occurs. The composition of these complexes shows that in the case of hydrochloric acid three-quarters of the total amino groups present form hydrochlorides, while in the case of picric acid half of the amino groups have formed picrates. That these acids do not stick so tightly to the amino group might be due to the stereochemical condition of the complex, to a weakening of the basic character of the amino group due to complex formation or to the fact that the acid character of the SH group is strengthened to such an extent in the complex that it forms a "zwitterion" with the amino group. That the acidity of the thiol group is very much augmented when the complex is formed can also be deduced from the fact that the free acid of III cannot be precipitated by acetic acid but

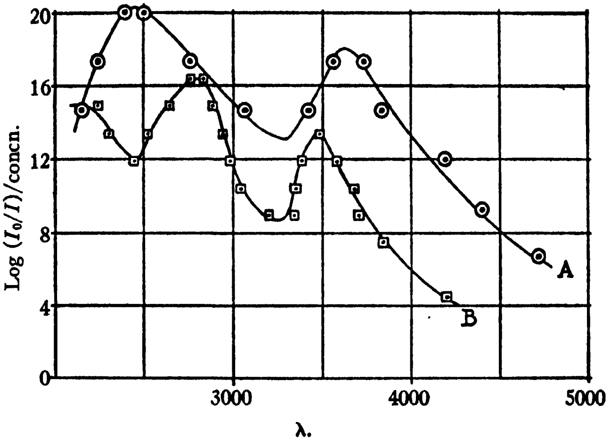

FIG. 1.—A, 0.075 millimolar potassium cobalti bis thioglycolate, B, 0.067 millimolar potassium cobalti bis cysteinate.

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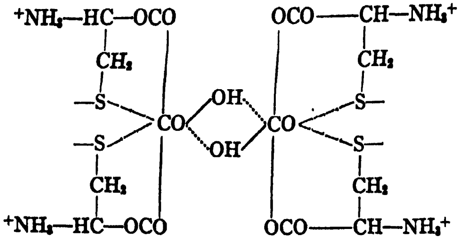
[5] G. F. Mann, *J. Chem. Soc.*, 2681 (1926); 890 (1928).

requires hydrochloric acid. If there were internal neutralization, the acid form of III would have the structure



and only the presence of excess of a strong acid would repress the ionization of the thiol group and permit the attachment of an acid at the amino group. At any rate, the fact that picrates and hydrochlorides can be formed at all is sufficient evidence that the amino group cannot be coördinated.

One further possibility remains. It seemed possible that the amino groups might be coördinated with the cobalt in alkaline solution but that as the solution is made acid there arises a competition of the cobalt and hydrogen ion for the free electron pair of nitrogen which results in sufficiently acid solution in the displacement of the cobalt from coördination with the amino group. As a matter of fact this is what seems to occur in the case of the alanine complexes, which are also more soluble in acid solution, but in this case with increasing acid there is a very marked color change. For example, on the addition of sufficient hydrochloric acid the violet color of the alanine complex in water changes to a red suggestive of cobalt chloride. On the other hand, the cobalti bis cysteinate complex does not change in color in hydrochloric acid solutions as concentrated as six molar and the ultraviolet absorption spectrum showed no change in two molar hydrochloric acid. The conclusion seems justified that at no P_H is the amino group coördinated with the cobalt.

The Constitution of the Cobalti Tris Cysteinate Complex.—The same problems arise with the complex IV containing three cysteine radicals for each cobalt atom. Since this complex is so insoluble in acids, it has not been possible to study the acid combining capacity of the amino groups; but a comparison of the absorption spectrum with that of the cobalti tris alanine complex shows, in spite of a similarity of composition, a great difference in the spectra (Fig. 2). There is also a difference in the visible part, the alanine complex showing a broad flat band at about 5300 to 5400 Å. and the cysteine complex showing only greatly increasing absorption toward the violet. It seems likely therefore that the cysteine complex involves coördination of three carboxyl and three thiol groups.

Thiohydracrylic acid, which differs from cysteine by the absence of the amino group, also forms cobalt complexes, but as with the other thiol acid complexes there has been considerable difficulty in isolating crystalline chemical individuals, so these complexes have not yet been studied.

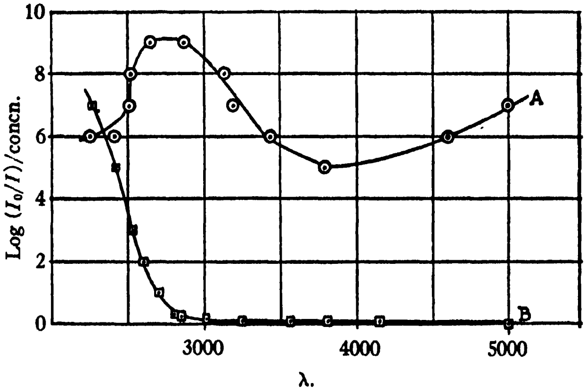


FIG. 2.—A, 0.1 millimolar potassium cobalti tris cysteinate; B, 0.1 millimolar cobalti tris alanine (violet form).

The Reaction of Hydrogen Peroxide with the Cobalto Bis Cysteinate Complex.—The peroxide reaction first noticed by Michaelis may be carried out as follows. If a dilute solution of a cobaltous salt, about 0.001 molar, is buffered at *P*H 7 to 8 with phosphate and excess cysteine is added, an olive-brown color is produced on shaking with air. If now a little dilute hydrogen peroxide solution, about 2%, is added, the color becomes an intense yellow. After a few hours this yellow gradually fades. In studying the properties of the complexes isolated, it was soon found that none of the oxidized complexes gives a reaction with hydrogen peroxide but that the blue-green potassium cobalto bis cysteinate does give the same intense yellow color reaction. However, after a solution of the blue-green complex has stood exposed to the air for some hours, it gives only a slight peroxide reaction. This suggested that the olive-brown complex described at the beginning of this paragraph gives the yellow complex with hydrogen peroxide only because the excess cysteine really keeps it partly in the reduced blue-green form and it is this part which gives the reaction. The blue-green color is very much less intense than the brown of the oxidized complex at the same molar concentration and could easily be masked by relatively smaller quantities of the latter.

To test out this idea 0.0002 *M* cobalt chloride solutions in thirtieth molar secondary phosphate were treated with different quantities of cysteine and the peroxide reaction tried before and after bubbling air through these solutions for various lengths of time. In some cases small amounts of iron salts were added to hasten the oxidation of the excess cysteine. It was found that if the molar ratio of cysteine to cobalt were less than four to one, the peroxide reaction was extremely faint. With larger excesses of cysteine, about twelve moles of cysteine per mole of cobalt, the peroxide reaction was quite intense, but after bubbling air through the solutions as described for fifteen hours a greatly diminished but undoubtedly positive reaction was given by the peroxide.

Work along these lines will be continued in this Laboratory, especially with a view to the isolation of the complex formed in the peroxide reaction as well as complexes it has been found possible to form with cystine by choosing the proper conditions. The complexes of cobalto bis cysteinate with carbon monoxide studied indirectly by Cremer[6] are also being taken up.

Preparation of the Unoxidized Complexes.—The unoxidized complexes were prepared in crystalline form, filtered, washed and dried in a stream of purified nitrogen using the apparatus sketched in Fig. 3.

Nitrogen freed from oxygen by passage through a tube packed with copper and kept at 450° enters the system through the wide test-tube A at the left. By successively adjusting the various stopcocks, this nitrogen is passed through all parts of the apparatus until air is completely washed out and the solutions deaërated. Test-tube A containing in solution the cysteine hydrochloride is then inverted by twisting in its rubber connections and the solution is pushed over into B by the nitrogen stream. B contains the necessary potassium hydroxide solution. It is then similarly inverted as A was and the alkaline cysteine solution is run into the flask C containing the cobalt chloride solution. Here the complex is formed in a reduced state. The flask I contains alcohol or any other precipitating agent which can be forced back into E by blowing into the outlet of I. The whole system A-B-C-D-F within the dotted area is rigidly connected and can be rotated about the axis indicated after freeing the rubber connections with flasks G and J. At D an alundum disk is fused into glass ground into the flask C at E. After turning upside down, the flask F is connected with J and the now upper outlet M of C is connected with G. At the same time the solution in C falls on the alundum disk and filters. If necessary, suction can be applied through K, closing stopcock L. After filtration is complete water in G and alcohol in H can be forced over successively into C by opening the stopcock M now connected to G. The stream of nitrogen is continued, which dries the precipitate on D. When ready the whole part D is taken out and quickly transferred to a desiccator, which is then evacuated. The dried crystalline compounds are stable in air.

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[6] W. Cremer, *Biochem. Z.*, **206**, 228 (1929).

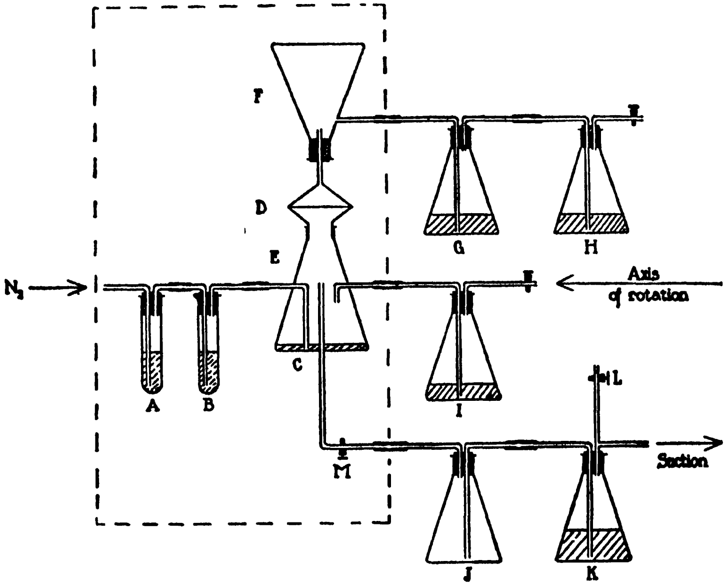

Fig. 3

Preparation of Potassium Cobalto Bis Cysteinate (*I*).—2.4 g. of cysteine hydrochloride dissolved in 10 cc. of water is put in A; 9 cc. of 7.5 *M* potassium hydroxide and 6 cc. of water are put in B; 7.5 cc. of 1 *M* cobalt chloride and 10 cc. of water in C. After deaëration and mixing as described above the flask C is warmed by bringing under it a beaker of hot water (M is closed and disconnected from J). Alcohol is forced over from I, about 60 cc., and the flask system C-D-F gently rocked and cooled. When cool the rigid system is inverted, the product sucked dry and washed with alcohol from G. After drying *in vacuo* over sulfuric acid, almost 2 g. of product is obtained, consisting of short stubby irregular blue-green needles; calculated for $Co(OOCCHNH_2CH_2SK)_2 \cdot 2H_2O$, Co, 14.35; N, 6.81; S, 15.57; K, 18.97. Found: Co, 14.60; N, 6.34; S, 15.66; K, 17.85.

Preparation of Cobaltous Cobalto Bis Cysteinate.—The grass-green cobalt salt II can be prepared in the same manner as the preceding compound using 14 cc. of the cobalt chloride solution and 6.5 cc. of potash in place of the quantities used there. It crystallizes out spontaneously, as it is quite insoluble. In fact it is so insoluble that it can even be prepared in the presence of air, but its preparation in the absence of air establishes the divalency of the cobalt.

Sixteen cc. of 7.5 *M* potassium hydroxide is poured into a solution of 10 g. of cysteine hydrochloride in 50 cc. of water and this mixture is poured into 60 cc. of 1 *M* cobalt chloride containing 16 cc. of 7.5 *M* potassium hydroxide. The whole is rapidly mixed, covered and set on ice. After half an hour the long, slender, grass-green needles have completely separated and are filtered off, washed with previously boiled water and then with alcohol. After drying in a vacuum over sulfuric acid, 8.2 g. is obtained; calculated for $Co(OOCCHNH_2CH_2S)_2Co \cdot 2H_2O$: Co, 30.10; N, 7.14; S, 16.32. Found: Co, 30.18; N, 6.85; S, 17.00.

Another preparation dried for only a few hours *in vacuo* over sulfuric acid gave: calculated for $Co(OOCCHNH_2CH_2S)_2Co \cdot 4H_2O$: Co, 27.56; N 6.54; S, 14.95. Found: Co, 26.96; N, 6.35; S, 15.09.

The percentage of cobalt precipitable by potassium hydroxide in the first preparation is 18.9%. This probably includes some due to decomposition of the complex by the strong alkali.

Preparation of the Cobalti-tris-cysteinate Complexes.—The simplest of the oxidized complexes to prepare is the free acid corresponding to IV. Ten cc. of 7.5 *M* potassium hydroxide in 15 cc. of water is added to a solution of 10 g. of cysteine hydrochloride in 40 cc. of water. This mixture is poured into 33 cc. of 1 *M* cobalt chloride containing 9 cc. of 7.5 *M* potassium hydroxide and the mixture quickly stirred and filtered. Air is bubbled through the mixture for an hour. After a day a heavy precipitate has settled out. Under the microscope this appears as opaque masses of platelets somewhat like bunches of grapes. It filters rapidly on a Büchner funnel and is washed with water several times and finally with alcohol. The product is dried by exposure to the air. Yields of about 90% of the theoretical can easily be obtained. It has not yet been found possible to recrystallize this acid. It is extremely insoluble in acids and water but dissolves in alkali giving cherry-red solutions. Calculated for $Co(OOCCHNH_2CH_2SH)_3 \cdot 3H_2O$: Co, 12.46; N, 8.88; S, 20.29. Found: Co, 12.50; N, 8.75; S, 20.06.

Another preparation which after standing for weeks was filtered and first washed with dilute alkali and dilute acid to remove any other complexes, cysteine or cobaltous hydroxide gave the following results: calculated for $Co(OOCCHNH_2$-

$CH_2SH)_3 \cdot 4H_2O$: Co, 12.01; N. 8.55; S, 19.55. Found: Co, 12.03; N, 8.36; S, 19.63.

The potassium salt of this complex has been made by dissolving 1 g. of the free acid in 25 cc. of water with 1.5 cc. of 7.5 *M* potassium hydroxide. The solution was evaporated somewhat and methyl alcohol and ethyl alcohol added in about equal parts until about 50 cc. had been added. Long red needle-like crystals separated. Calculated for $Co(OOCCHNH_2CH_2SK)_3 \cdot 2H_2O$: Co, 10.37; N, 7.38; K, 20.57. Found: Co, 10.21; N, 7.47; K, 20.25.

The conditions under which these latter crystals are formed have not been fully worked out and it frequently happens that the product obtained instead of being crystalline is amorphous or liquid. This complex gives extremely insoluble alkaline earth salts which have not been crystallized.

Preparation of the Cobalti-bis-cysteinate Complexes.—The free acid corresponding to the oxidized complex III has been prepared, starting with both bivalent and trivalent cobalt. Either procedure gives the same crystalline product in about 50–60% of the theoretical yield. Only the procedure starting with bivalent cobalt need be described. Five grams of cysteine hydrochloride is dissolved in 20 cc. of water and 7.5 cc. of 7.5 *M* potassium hydroxide is added. This solution is poured into 16 cc. of 1 *M* cobalt chloride containing 4.5 cc. of 7.5 *M* potassium hydroxide. The mixture is stirred and filtered into a flask and air is sucked through for about an hour. Then concentrated hydrochloric acid is added drop by drop until the amorphous precipitate first formed redissolves or crystallization begins. Then 50 cc. of 95% alcohol is added and the whole set on ice. The crystals usually come out in the form of hexagonal plates and complicated patterns of these, but occasionally the crystal shape was altogether different. They are filtered, washed with a little cold water and then with alcohol. The yield after drying by exposure to the air is 3.7 g. The product may be recrystallized by dissolving in potassium hydroxide and reprecipitating with hydrochloric acid and alcohol. The mean of results from several independent preparations follows. Calculated for $[Co(OOCCHNH_2CH_2SH)_2OH]_2 \cdot 8H_2O$: Co, 15.20; N, 7.22; S, 16.50. Found: Co, 15.39; N, 7.26; S, 16.99.

The potassium salt of this complex was obtained by following the above procedure through the sucking of air through the mixture for one hour and then alcohol is slowly added till precipitation begins. The crystallization of this potassium salt is, like that of the potassium tris-cysteinate complex previously described, subject to undetermined factors which frequently lead to amorphous or liquid precipitates. The latter usually form if the solution is too alkaline. The analysis of a crop of needle-like brown crystals growing in dense sheaves is given. Calculated for $[Co(OOCCHNH_2CH_2S_2)HK(OH)]_2 \cdot 4H_2O$: Co, 15.10; N, 7.18; S, 16.42; K, 10.01. Found: Co, 15.29; N, 7.49; S, 16.22; K, 9.35.

The corresponding barium salt has also been crystallized by starting with the free acid previously described, dissolving in water with the aid of a little potassium hydroxide and adding barium chloride solution. The crystals are cubical. Calculated for $[Co(OOCCHNH_2CH_2S)_2H(OH)]_2Ba \cdot 9H_2O$: Ba, 14.75; Co, 12.70; S, 13.78; N, 6.01; H_2O, 19.38. Found: Ba, 14.70; Co, 12.71; S, 14.01; N, 5.73; H_2O 19.26.

This is the only one of the cysteine complexes in which water could be determined by heating to constant weight at 110°, all the others slowly undergoing decomposition during which nitrogen is lost. Water could not well be determined in a vacuum over sulfuric acid as in most cases even after weeks no definite end-point could be found.

The free acid of this series dissolves only slightly in water but readily on addition of hydrochloric acid. From sufficiently strong hydrochloric acid long brown needle-shaped crystals of a hydrochloride deposit and are stable so long as the acid concentration is maintained high enough. Preparations were made as follows. Two grams of the free acid is dissolved in 15 cc. of water with 15 cc. of concentrated hydrochloric acid and 5 to 10 cc. of alcohol is added. The mixture is set on ice for an hour or two, then filtered on a Büchner funnel and sucked as dry as possible. The compound cannot be washed with water or alcohol as it readily loses hydrogen chloride, giving the free acid again. Drying in a vacuum over concentrated sulfuric acid causes dissociation of considerable hydrogen chloride, as is evident on opening the desiccator. Analysis of the compound so dried gave these figures: calculated for $[Co(OOCCHNH_2CH_2SH)_2OH]_2 \cdot 3HCl \cdot H_2O$: Co, 15,54; N, 7.38; Cl, 14.03. Found: Co, 15.71; N, 7.12; Cl, 14.10.

The corresponding picrate was prepared by dissolving 1.7 g. of the free acid in 60 cc. of water with the aid of 10 cc. of concentrated hydrochloric acid and then adding 150 cc. of saturated picric acid. A crystalline precipitate consisting of brown needles and a few square plates settled out. This, like the hydrochloride, could not be washed but was merely sucked as dry as possible. After drying in a vacuum over concentrated sulfuric acid, 3 g. was obtained. The analyses corresponded to $[Co(OOCCHNH_2CH_2SH)_2]C_6H_2(NO_2)_3OH \cdot 9H_2O$. Calculated: Co, 8.56; N, 10.15; S, 9.29. Found: Co, 8.58; N, 11.64; S, 9.64.

The very high nitrogen content is probably due to the fact that the precipitate could not be washed, and retained some of the mother liquors containing excess picric acid.

Oxygen consumption studies on the formation of the oxidized complexes similar to those described in the study of the thioglycolic acid complexes show that one equivalent of oxygen is taken up for each mole of cobalt.

SUMMARY

A number of cobalt complexes of cysteine in both reduced and oxidized states have been crystallized and their chemical constitutions, properties and interrelationships discussed.

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SOME ULTRAVIOLET PHOTOMICROGRAPHS OF B. SUBTILIS

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(*From the Laboratories of The Rockefeller Institute for Medical Research*)

Plates 42 to 44

(Received for publication, May 29, 1931)

In the course of some studies being made with the ultraviolet microscope we have prepared photographs of *B. subtilis* which yield useful information concerning the internal structure of this bacterium and the way spores arise within it.

Two bacterial strains were examined. One was non-sporulating and came from the Type Collection in Chicago; the other, which grew larger bacilli and readily produced spores in old cultures, was isolated in this laboratory. Before being photographed the living unstained cells were transferred to the surface of a thin layer of NaCl-agar spread upon a quartz slide. In this way they were fixed in position and kept stationary during exposure.

Technique

The microscope was a modification of the instrument designed by Barnard[1] and manufactured by Beck. Its optical parts, as used in the present experiments, were, however, quartz objectives and eyepieces by Zeiss. As their design requires, these objectives have been employed with light of wave length 2750 A. Desired objects are found with the green line of the mercury arc; approximate focus in the ultraviolet is then achieved by applying a precise but arbitrarily determined correction. The focal plane is shallower with ultraviolet than with visible light and it is usually necessary to make a series of exposures at different depths through a preparation. This has been done by taking photographs upon motion picture film. The small Leica camera modified by removing its glass optical parts and giving it a very rigid support behind the eyepiece of the microscope is convenient for this purpose. Ordinary positive film gives excellent results—it is sufficiently rapid in the ultraviolet and its fine grain permits very satisfactory enlargements. The accompanying photographs have thus been made, the objective being the standard Zeiss 1.70 mm. glycerine immersion monochromat. Exposure times varied from ¼ to 3 seconds.

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[1] Barnard, J. E., *Lancet*, 1925, **2,** 117.

Ultraviolet microscopy has two outstanding advantages over that using visible light. One resides in the greater resolving power which is a consequence of the short wave length; the other arises from the fact that, since some proteins absorb in the ultraviolet more strongly than others, it is often possible to see detail in living and unstained cells. Both of these advantages are realized in the accompanying pictures. The first figure, of a 5 hour culture on agar, makes it clear that young and rapidly growing cells contain no observable structures. In older cultures of both the non-sporing and the sporing lines cells become granulated (Figs. 3–6). At first these granules are few in number and small. Very old bacilli, however, are often filled with inclusions of all sizes (Fig. 2). *It is important to notice that in none of these photographs is there any indication of formed nuclei.*

Especially interesting is the evidence bearing upon spore production. These bodies are intensely absorbing (Figs. 2–5) and many stages in their development can therefore be seen directly. They do not arise through the gradual accumulation and merging together of the refractile granules or of any preexisting cell bodies that have strong ultraviolet absorption. Instead, at the first appearance they have their final size and shape. In initial stages they are only faintly absorbing but as they develop this absorption becomes progressively greater (Fig. 4). Cells containing mature spores are approximately as opaque as other bacteria. This suggests that spores when fully formed are not a mere condensate of a bacterium's protoplasmic or chromatin content.

There are three frequently expressed views concerning the origin of spores.[2] According to one they arise after a nuclear division which has partitioned the chromatin between a spore-anlage and the cell nucleus. Other investigators have described them as resulting from the coalescence of chromatin granules which in more recent work are definitely associated with nuclei thought to be present. It is apparent that the spores of *B. subtilis* do not have the small beginnings required by this hypothesis. A third idea makes spores the result of a more or less gradual concentration of cell substance. Some have thought that this concentration was in the main a dehydration succeeded by the infiltration of lipoids. In its broad outlines such an hypothesis is not incompatible with our photographs. At present ultraviolet microscopy is obviously unable to interpret chemical changes that may take place but nevertheless pictures such as Fig. 4 seem to demand a mechanism more elaborate than mere dehydration.

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[2] See for example Gotschlich, E., in Kolle, W., Kraus, R., and Uhlenhuth, P., Handbuch der pathogenen Mikroorganismen, Jena, Gustav Fischer, and Berlin and Vienna, Urban and Schwarzenberg, 3rd edition, 1929, 1, 33.

EXPLANATION OF PLATES

PLATE 42

FIG. 1. A young culture of *B. subtilis* growing on agar, photographed 5 hours after inoculation and 10 minutes after being covered with quartz. 3300 ×.

FIG. 2. Cells from a 6 day agar culture transferred to a film of fresh nutrient agar just before being photographed. The large inclusions present in the degenerating cells can easily be seen. 2500 ×.

PLATE 43

FIG. 3. Cells of a 31 hour old culture of non-sporulating *B. subtilis* growing on agar. The inclusions present in nearly every cell are clearly visible. 3500 ×.

FIG. 4. Cells from a 20 hour culture of spore-forming *B. subtilis.* The bacteria were transferred to a film of pure agar immediately before being photographed. Different cells contain spores in all stages of development (and of corresponding opacity). 3500 ×.

PLATE 44

FIG. 5. Bacteria from a 24 hour culture of sporulating *B. subtilis* grown on agar. They were transferred before being photographed to a film of 2 per cent agar in 0.85 per cent NaCl on a quartz slide. It will be noticed in this and the preceding picture that, though spores do not grow from granules, cells containing mature or developing spores are relatively free from inclusions. 3500 ×.

FIG. 6. The same field as the preceding but with the focal plane slightly above that of the bacteria. 3500 ×.

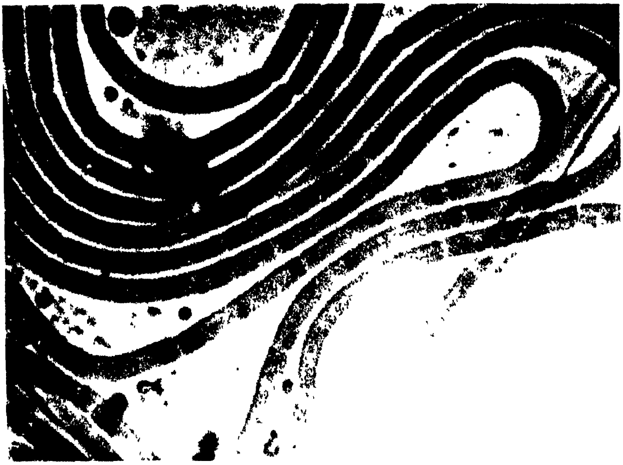

FIG. 1

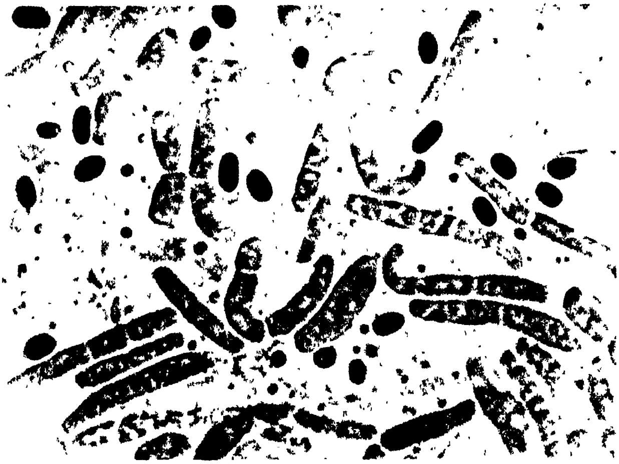

FIG. 2

(Wyckoff and Ter Louw: Ultraviolet photomicrographs of *B. subtilis*)

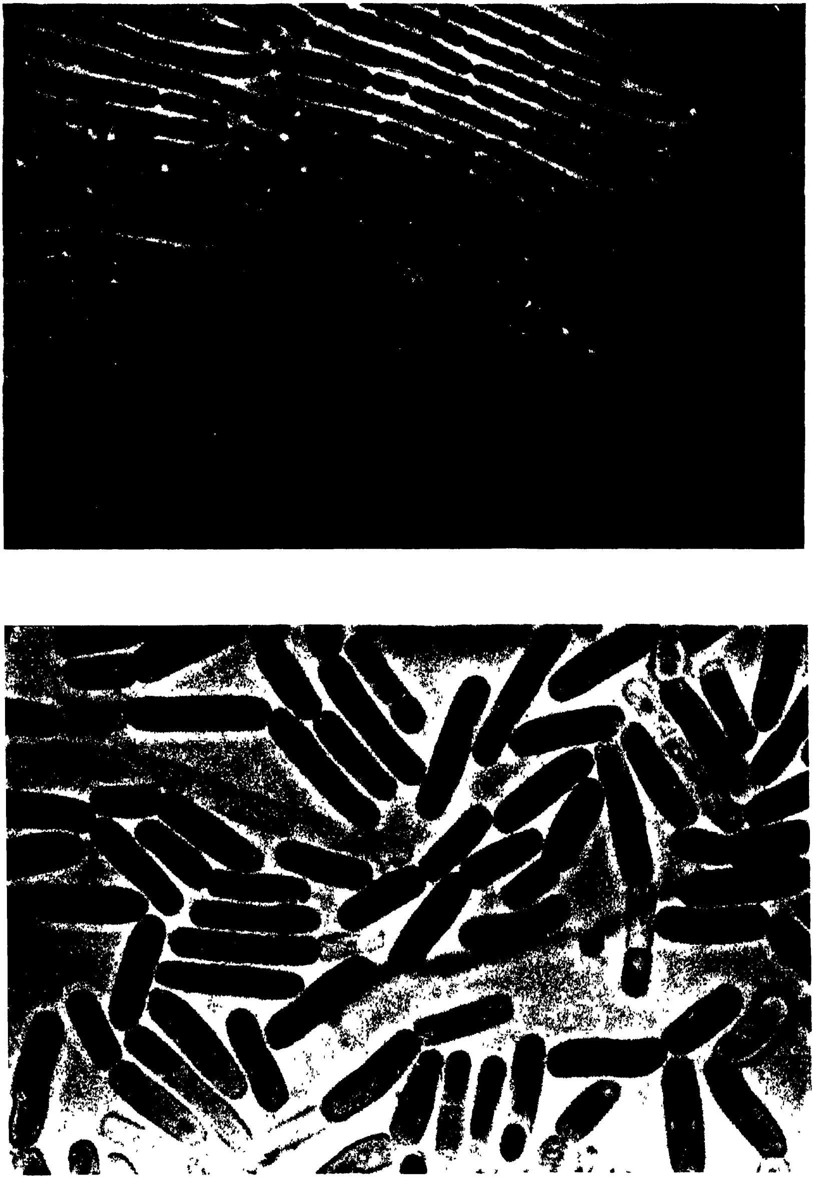

Fig. 4

(Wyckoff and Ter Louw: Ultraviolet photomicrographs of *B. subtilis*)

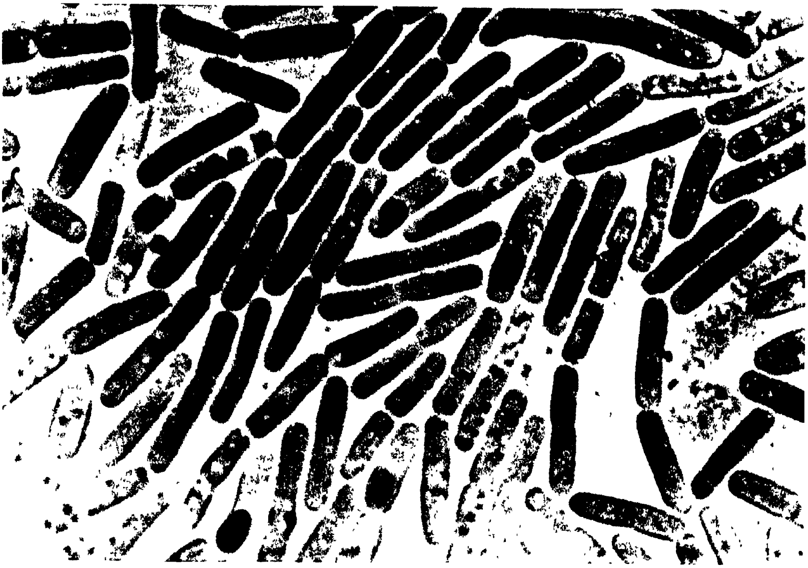

Fig. 5

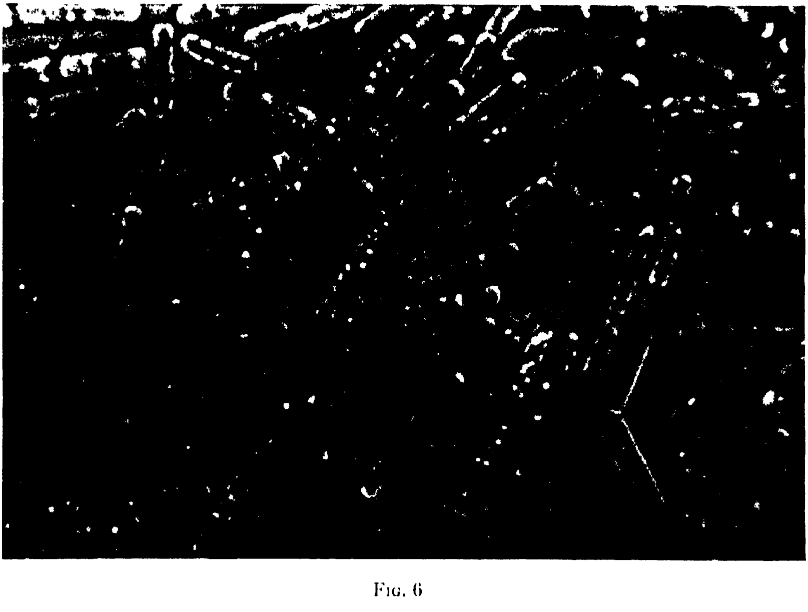

Fig. 6

(Wyckoff and Ter Louw: Ultraviolet photomicrographs of *B. subtilis*)

[Reprinted from ZEITSCHRIFT FÜR KRISTALLOGRAPHIE, September, 1931, Vol. 80, Nos. 1–2, pp. 1–4]

THE X-RAY INVESTIGATION OF CERTAIN DERIVATIVES OF CYCLOHEXANE

IV. *i*-INOSITOL DIHYDRATE

BY T. N. WHITE[1]

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, May 9, 1931)

In the preceding paper of this series[2]) the space group of *i*-inositol has been discussed. For reasons there given it was necessary to base the identification on the properties of its dihydrate. A fairly thorough examination of this latter substance was therefore necessary. On account of its instability the dihydrate is a rather unsatisfactory subject for X-ray investigation, but certain similarities in the general crystalline form of the two substances made it worth while to attempt at least a determination of its space group. It may be noted that the published morphological data on *i*-inositol dihydrate give no indication of any relation with the axes of the anhydrous form, while the new setting for the dihydrate here given clearly shows that such relations do exist. Since the results of this paper have already[3]) been compared with those for *i*-inositol, no further mention of this question will be made herein.

The dihydrate of *i*-inositol has been examined crystallographically by several observers, with results summarized from Groth[4]) as follows:

density: 1.524 gm. cm.^{-3}
crystal class: monoclinic prismatic
$a : b : c = 1.0105 : 1 : 0.3910$
$\beta = 90° 37'$.

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1) National Research Fellow (U.S.A.).

2) T. N. White, Z. Krist, 78, 91. 1931. The Hermann symbol for *i*-inositol is incorrectly given. It should read $C_{2h}p2$.

3) T. N. White, loc. cit.

4) Chem. Kryst. 3, 609.

The general appearance of the crystals resembles that of *i*-inositol in that both form very thin elongated tablets $\{010\}$; they differ markedly in that the dihydrate crystals have excellent faces, and that much larger specimens can easily be grown.

Observation of the edge angles, and angle of extinction, under the microscope gave data in excellent agreement with those of Groth. The fact that the indexing of all the X-ray photographs was successfully carried out on the basis of one axial translation determined by X-rays, the remaining elements being calculated from the crystallographic data, provides the strongest evidence that the substance under investigation is really the dihydrate of *i*-inositol.

On account of the fact that the crystals effloresce under normal atmospheric conditions, considerable difficulty was experienced in getting the desired X-ray data. Several attempts at coating specimens with different substances (paraffin, shellac) and even embedding them in pellets of wax and cement did not noticeably prolong their lives, and the method finally adopted was that of using a newly set up (Shearer) X-ray tube with a good focus, and a fresh crystal for nearly every photograph.

One axis (*b*-axis) was determined by X-rays, and the other two were calculated from it by the morphological axial ratios. The indexing which was carried out on this basis gave the following groups of planes of type $\{hkl\}$:

19 present with	$(k + l)$	odd,
0	$(l + h)$	,
19	$(h + k)$	,
20	$(h + k + l)$	, .

so that evidently the (010)-face of this cell is centréd, and the crystallographically chosen axes do not lead to a primitive triplet. The axes were therefore changed so that:

new a = old $[10\bar{1}]/2$ = 8.98 Å
b = b = 16.59 Å
c = c = 6.49 Å
β = 109.8°

$$\begin{aligned} \text{Cell volume} &= 902\ \text{Å}^3 \\ C_6H_{10}O_8 \text{ per cell} &= 4 \\ \text{Density} &= 1.58\ \text{gm. cm.}^{-3}. \end{aligned}$$

The 4% discrepancy in the two densities is not surprisingly large when it is considered that two of the cell dimensions were calculated instead of being directly measured.

Re-indexing on the basis of the new axis, it was found that, of planes of type $\{hkl\}$ there were:

11 present with	h odd,
14	l " ,
16	$(k + l)$ " ,
19	$(l + h)$ " ,
24	$(h + k)$ " ,
17	$(h + k + l)$ " ,

so that the cell based on these axes is primitive and the lattice simple monoclinic Γ_m.

The indexing of several photographs obtained by oscillation about the b-axis gave the following information:

Planes of type $\{h0l\}$
- 0 present with h odd, (27) out of 30 absentees with h odd)
- 10 l ,
- 10 $(h + l)$,

Planes of type $\{0k0\}$
- k = 2, 4, 6, present,
- k = 1, 3, 5, absent.

It can therefore be concluded that

$\{h0l\}$ halved if h is odd,
$\{0k0\}$ halved,

are very probably true space group halvings. If the prismatic classification of the crystal is correct, the space group is most likely

$$C_{2h}^5\ (C_{2h}pa2).$$

Choosing, for the purpose of description, a convenient origin at the intersection of a screw axis with glide plane, the disposal of asymmetry elements consistent with the above space group, and axes of the crystal as chosen, will be as follows:

Centres of symmetry at the intersections of the planes

$$x = \tfrac{1}{4}, \tfrac{3}{4};\ y = \tfrac{1}{4}, \tfrac{3}{4};\ z = 0, \tfrac{1}{2}.$$

Glide planes: $y = 0, \tfrac{1}{2}$; translation τ_x.

Digonal screw axes: intersections of the planes

$$x = 0, \tfrac{1}{2};\ z = 0, \tfrac{1}{2}.$$

The co-ordinates of the four equivalent points are in this case:

$$x, y, z;\ x + \tfrac{1}{2}, \bar{y}, z;\ \bar{x}, y + \tfrac{1}{2}, \bar{z};\ \tfrac{1}{2} - x, \tfrac{1}{2} - y, \bar{z};$$

and as there are four molecules in the cell, the space group requirements do not impose upon them any symmetry.

SUMMARY

i-Inositol dihydrate crystallizes in the prismatic class of the monoclinic system. Its cell dimensions, in a new setting are:

$a = 8.98$ Å $\beta = 109.8°$
$b = 16.59$ Å
$c = 6.49$ Å Lattice T_m.

The most probable space group is C_{2h}^5 ($C_{2h}pa2$) with four molecules in the elementary cell.

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THE X-RAY INVESTIGATION OF CERTAIN DERIVATIVES OF CYCLOHEXANE

V. α- AND γ-CYCLOHEXANDIOL 1.2, β-CYCLOHEXANDIOL 1.4 (TRANS-QUINITOL), AND β-CYCLOHEXANDIACETATE 1.4

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(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, May 9, 1931)

In previous papers²) of this series there have been determined the space groups of a cyclohexanpentol, two cyclohexanhexols, and certain associated derivatives. Before attempting a more detailed analysis of these crystals it was considered advisable to investigate the cyclohexandiols, in order to discover whether among them there might be some one which would appear more promising for structure analysis than was found among the higher substituted derivatives. As will be shown herein, it does seem that two such cases have been found though it is difficult yet to make a positive statement to that effect.

Two ortho and two para cyclohexandiols have been known for some time. The meta derivatives of cyclohexane do not appear to have been investigated to any extent until comparatively recently³). Owing to controversy as to which of the ortho isomers is the "cis," and which the "trans" form, all substances will be referred to in the following by their Greek letter nomenclature.

The earliest thorough chemical investigation of the ortho cyclohexandiols seems to be due to Brunel⁴) who took up the work from Markownikow's isolation of the α-compound⁵), obtained the β-isomer, and studied the properties of a number of derivatives involving radicals

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1) National Research Fellow (U. S. A.).

2) A. L. Patterson and T. N. White, Z. Krist. **78**, 76, 86. 1931; T. N. White, ibid. **78**, 91. 1931 and preceding paper in this issue.

3) Beilstein, Handb. org. Chem. **6**, 740–41.

For recent chemical investigations on the cyclohexandiols, see, for example, B. Rothstein, Ann. Chim. **14**, 461. 1930.

4) Ann. Chim. (8) **6**, 200. 5) Lieb. Ann. **302**, 22.

other than the hydroxyl group. It has not been possible completely to identify the β-isomer upon which the X-ray examination has been made with the substance described by Brunel, and it has therefore been thought advisable to provide an additional designation (γ) which may serve to distinguish between the two until the matter has been settled.

The para cyclohexandiols and the corresponding diacetates were first isolated and studied by Baeyer[1]). All four of these substances were prepared for the X-ray examination, but only the results on the two β-derivatives are given here. Repeated attempts failed to give good crystals of the α-diol. Its investigation, and that of the corresponding acetyl derivative which requires cold weather for preservation in the solid state, have been postponed. As the writer will not be able to undertake these tasks immediately, and as two of the four substances here considered have given results of exceptional interest, it has seemed best to put on record the information now on hand.

The experimental procedure has been essentially the same as that outlined in previous papers of this series. A careful comparison has been made in the case of each substance with properties given in the literature, to ensure that its identity is correct. In some cases a crystallographic examination has been made to supply necessary data.

α-Cyclohexandiol 1.2

This substance, first prepared by Markownikow[2]), was described by Brunel as a "trans" compound. Evidence has been brought forward since then for changing it to the "cis" classification[3]). Its crystallographic properties have been studied[4]) and it has been assigned to the orthorhombic bipyramidal class, with axial ratios:

$$a : b : c = 0.8868 : 1.1393.$$

The material used for this investigation was prepared by R. E. Marker of this laboratory according to the method of Markownikow. Its melting point was found to be 97–98°C., in good agreement with

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1) Ber. Dtsch. chem. Ges. **25**, 1037, 1840. 1892; Lieb. Ann. **278**, 92. 1894.

2) loc. cit.

3) Derx, Rec. Trav. chim. **41**, 334. 1922; P. Bedos, C. r. **183**, 750. 1926.

4) Groth, Chem. Kryst. **3**, 606.

the value 99–100°C. of the literature. Good crystals were obtained from a mixture of alcohol and benzene, and examination under the microscope showed that they exhibited the proper form and edge angles, and optical properties. By suspension in the appropriate mixture of alcohol and ethyl bromide, their density was determined as 1.182 gm. cm.$^{-3}$ at 25°C.

The X-ray photographs all showed the symmetry to be expected from orthorhombic crystals. From the layer line spacings on oscillation photographs obtained with accurately set crystals[1]) the axial translation of the unit cell were calculated, yielding the following results.

$a = 7.62$ Å Cell volume $= 1280$ Å^3
$b = 8.55$ Å $C_6H_{12}O_2$ per cell $= 8$
$c = 19.57$ Å Density $= 1.20$ gm. cm.$^{-3}$
$a : b : c : = 0.892 : 1 : 2.29.$

Evidently the c-axis of the morphological examination should be doubled.

It may be remarked that due to their high vapor pressure small crystals of this substance evaporate away in a few days, so that settings cannot be kept for any length of time and the photographs must be taken as soon as possible.

From the indexing of two oscillation photographs the following data were gathered for the purpose of determining the lattice upon which the structure is based:

Planes of type $\{hkl\}$ with $(k + l)$ odd: 21 present,
$(l + h)$: 23 ,,
$(h + k)$: 25 ,,
$(h + k + l)$: 20 ,,

Clearly the cell is neither body nor face centred, and the lattice is therefore simple orthorhombic, Γ_0.

---

1) The goniometer heads on which the crystals were mounted were interchangeable between the X-ray spectrograph and an ordinary morphological crystallographic goniometer, so that excellent settings were obtainable with crystals which possessed a few good faces.

Due to the weakness of a surprisingly large number of reflections from critical planes for space group determination, it was found necessary to take three series of oscillation photographs (two about the c-axis and one about the b-axis) in order to firmly establish the following data:

$$\text{Planes of type } \{0kl\} \begin{cases} 4 \text{ present with} & k \quad \text{odd} \\ 0 & l \\ 4 & (k+l) \end{cases} ,$$

$$\text{Planes of type } \{h0l\} \begin{cases} 0 \text{ present with} & h \quad \text{odd} \\ 8 & l \\ 8 & (h+l) \end{cases} ,$$

$$\text{Planes of type } \{hk0\} \begin{cases} 5 \text{ present with} & h \quad \text{odd} \\ 0 & k \\ 5 & (h+k) \end{cases} .$$

The data appear to be somewhat scanty, but in view of the fact that they represent with regard to most of the planes the concordant results of three series of photographs, on the last series of which the exposures were considerable, and as in no case did discordant results appear it is fairly safe to conclude that the following are true space group halvings of the crystal:

$\{0kl\}$ if l odd; $\{h0l\}$ if h odd; $\{hk0\}$ if k odd.

An examination of the Astbury and Yardley¹) space group tables shows that the only orthorhombic space group based on Γ_0 which provides these halvings is V_h^{15} ($D_{2h}p\gamma\alpha\beta$, symbols arranged to agree with the observed glide translations). This space group is illustrated in Fig. 61 of the tables mentioned above. If as in the figure we adopt a centre of symmetry as origin, there will be the following symmetry elements associated with the cell:

Centres of symmetry at the corners, edge centres, face centres and body centre of the cell.

Glide planes: $x = \frac{1}{4}, \frac{3}{4}$; translation τ_z
$y = \frac{1}{4}, \frac{3}{4}$; τ_x
$z = \frac{1}{4}, \frac{3}{4}$; τ_y

---

1) W. T. Astbury and K. Yardley, Phil. Trans. (A) 124, 221. 1924.

Digonal screw axes: intersections of planes $y = 0;\ z = \frac{1}{4}, \frac{3}{4}$

$z = 0;\ x = \frac{1}{4}, \frac{3}{4}$

$x = 0;\ y = \frac{1}{4}, \frac{3}{4}$.

As there are 8 molecules in the cell, they may possess general positions. In order to obtain the co-ordinates of the equivalent points of this space group consistent with the axes as chosen for the crystal, the y and z co-ordinates of the Wyckoff tabulation must be interchanged. We then obtain:

$$x, y, z;\ \tfrac{1}{2} - x, y, z + \tfrac{1}{2};\ x + \tfrac{1}{2}, \tfrac{1}{2} - y, z;\ x, y + \tfrac{1}{2}, \tfrac{1}{2} - z;$$
$$\bar{x}, \bar{y}, \bar{z};\ x + \tfrac{1}{2}, \bar{y}, \tfrac{1}{2} - z;\ \tfrac{1}{2} - x, y + \tfrac{1}{2}, \bar{z};\ \bar{x}, \tfrac{1}{2} - y, z + \tfrac{1}{2}.$$

γ-*Cyclohexandiol* 1.2

As previously stated, this substance was first prepared by Brunel¹) and wrongly, according to recent investigations (vide supra), classified by him as a "cis" compound. Many of its properties are similar to those of the α-modification²). The following crystallographic data are on record³):

Crystal class: orthorhombic bipyramidal.
$a : b : c = 0.9424 : 1 : 1.1370$.
Thin tablets {001} with edge faces
$o\{111\}$, $x\{221\}$, $q\{011\}$.
Good cleavage {001}.

The melting point has been given as 104°C.

The substance used for this investigation was prepared by R. E. Marker, of this Institute, according to the method of Brunel⁴). Examination under the microscope showed, however, that in both form and optical properties the crystals were monoclinic and not orthorhombic. The monoclinic symmetry was corroborated by a Laue photograph.

The preparation was therefore repeated by Mr. Marker using several methods, one of which was that of Brunel as exactly as it could be fol-

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1) loc. cit.

2) Beilstein, Handb. org. Chem. 6, 740.

3) Groth, Chem. Kryst. 3, 607.

4) ibid. pp. 236, 219.

lowed from his record. All methods yielded the same substance. A combustion analysis gave the following composition:

	Observed	$C_6H_{12}O_2$
Carbon	62.3 %	62.1 %
Hydrogen	10.41%	10.34%

and the melting point was determined as 104°C. These data *verify the percentage constitution, and preclude the possibility of the compound being the "equimolecular mixture" of the α- and β-forms, which* has a melting point of about 72°C.[1]). It may be also noted here that the molecular weight is in accordance with the X-ray data (vide infra).

Since the crystals were obtained from a one to one (by volume) mixture of alcohol and benzene, as were those of Brunel[2]) which were presumably supplied to Wyrouboff for the crystallographic examination, there seems to be no apparent reason for the appearance of a different form. Crystallization from benzene and alcohol separately also gave the monoclinic form. There is of course the bare possibility that γ-cyclohexandiol 1.2 might be one of the optical antipodes into which the β-preparation of Brunel was separated by Derx[3]). Examination showed, however, that the substance of this examination has no detectable optical activity, so that this explanation cannot be accepted. To summarize, then the weight of evidence is in the favor of the identity of β- and γ-cyclohexandiol 1.2. Nevertheless, if Wyrouboff's data apply to the β-form, and not through some accidental error to some other substance, it must be concluded that γ is a distinct form. Therefore, since the accidental error, if there was one, can only be proven by the discovery of the substance to which the Wyrouboff data apply, the present distinction between the β- and γ-forms is the only safe course to follow.

An examination of the γ-cyclohexandiol 1.2 on the goniometer provided the following data:

Crystal class: monoclinic prismatic.
$a : b : c = 1.954 : 1 : 0.716$
$\beta = 103.9°$
Forms: *a* {100}, *p* {011}, *r* {110}, *x* {211}.

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1) Beilstein, loc. cit. 2) ibid. p. 247. 3) loc. cit.

The crystals (from benzene and alcohol) are usually in the form of thick tablets a $\{100\}$ with the greatest dimension in the direction of the c-axis. Like the α-form, they have a high vapor pressure, and the faces spoil rapidly if the crystals are not kept bottled up. The density is 1.147 gm. cm.$^{-3}$ at 24°C.

The cell data, determined from X-ray oscillation photographs about the same axes as used for the morphological examination, are:

$a = 19.13$ Å Cell volume $= 1332$ Å^3
$b = 9.92$ Å $C_6H_{12}O_2$ per cell $= 8$
$c = 7.23$ Å Density $= 1.157$ gm. cm.$^{-3}$
$a:b:c = 1.930:1:0.729.$

Two oscillation photographs were completely indexed for the purpose of determining the lattice type. The results follow.

Planes of type hkl with $(k + l)$ odd: 18 present,
$(l + h)$: 21 ,
$(h + k)$: 18 ,
$(h + k + l)$: 0 .

Evidently the crystal is based on the Γ_m' monoclinic lattice, and the axes have been so chosen that the cell is body centred. Indexing of all subsequent photographs gave no inconsistency with this result. A series of ten 20° oscillation photographs about [010] was then examined to determine the space group halvings. From these it was found that no planes of type $\{h0l\}$ reflect in the cases: h odd, l odd, $(h + l)$ odd, though some twenty of each had an opportunity to record on the plate. 12 planes of the same type, but with both indices even, were present. These data account for all but one or two planes which came within the range of observation. We can therefore conclude with a considerable degree of certainty that the space group is such that planes $\{h0l\}$ only reflect if both indices are even. The monoclinic space groups based on Γ_m' which are consistent with this property are C_s^4 and C_{2h}^6. If the assignment of the crystal to the holohedral class is correct, then the space group is C_{2h}^6 $(C_{2h}b\alpha 1)$. This is No. 15 of the Astbury and Yardley Tables. If the origin is taken in a digonal axis at such a point that the plane $y = 0$ may contain centres of symmetry, then the symmetry elements associated with an unit cell may be described as follows:

Centres of symmetry at the intersections of the planes:

$$x = \tfrac{1}{4}, \tfrac{3}{4};\ y = 0, \tfrac{1}{2};\ z = 0, \tfrac{1}{2};$$
$$x = 0, \tfrac{1}{2};\ y = \tfrac{1}{4}, \tfrac{3}{4};\ z = \tfrac{1}{4}, \tfrac{3}{4}.$$

Glide planes: $y = 0, \tfrac{1}{2}$; translation τ_x

$y = \tfrac{1}{4}, \tfrac{3}{4}$; τ_z.

Digonal axes: intersections of the planes:

$$x = 0, \tfrac{1}{2};\ z = 0, \tfrac{1}{2}.$$

The eight molecules in the cell may possess general positions. In order to make the Wyckoff tabulation for this space group consistent with the axes as chosen for this crystal, the co-ordinates of the equivalent points must be changed to apply to a body centred cell, with the *b*-axis digonal; we then obtain:

$$x, y, z;\ \bar{x}, y, \bar{z};\ x + \tfrac{1}{2}, \bar{y}, z;\ \tfrac{1}{2} - x, \bar{y}, \bar{z};\ x + \tfrac{1}{2}, y + \tfrac{1}{2}, z + \tfrac{1}{2};$$
$$\tfrac{1}{2} - x, y + \tfrac{1}{2}, \tfrac{1}{2} - z;\ x, \tfrac{1}{2} - y, z + \tfrac{1}{2};\ \bar{x}, \tfrac{1}{2} - y, \tfrac{1}{2} - z.$$

β-Cyclohexandiol 1.4

(*trans-Quinitol*)

Both the α- and β-substances and their diacetates were prepared by R. E. Marker, of this laboratory, from the *cis*-quinitol of the Eastman Chemical Laboratories by a method principally due to Baeyer[1]). The melting point of all four substances were found to agree well with those in the literature, so that the identity of the compounds used for this analysis may safely be regarded as established.

No crystallographic data are, to the writer's knowledge, on record. Crystals were first obtained from a mixture of alcohol and benzene, and later from acetone, and also from 95% alcohol. All exhibited nearly the same forms.

Crystal class: monoclinic prismatic.

$$a : b : c = 0.293 : 1 : 0.339$$
$$\beta = 96°.$$

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[1]) Lieb. Ann. **278**, 92. 1894.

Forms observed (from alcohol and benzene):

$b\ \{010\}, q\ \{101\}, x\ \{110\}, m\ \{011\}, r\ \{130\}, p\ \{031\}$.

Tablets $\{010\}$.
The density was found to be 1.18 gm. cm.$^{-3}$ at 20°C.

The X-ray photographs showed the symmetry to be expected of monoclinic crystals. Adopting the same axial direction as were used in the crystallographic examination the following cell data were obtained:

$a = 6.32$ Å Cell volume $= 968$ Å^3
$b = 21.2$ Å $C_6H_{12}O_2$ per cell $= 6$
$c = 7.27$ Å Density $= 1.19$ gm. cm.$^{-3}$
$a:b:c = 0.298:1:0.343$.

From the indexing of two oscillation photographs it was found that reflections occurred from the following groups of planes of type $\{hkl\}$:

28 with $(k + l)$ odd,
37 $(l + h)$,
33 $(h + k)$,
30 $(h + k + l)$.

The cell is therefore neither face nor body centred. The presence of six molecules in the cell must now be explained; this may be due to either of the following two causes, or to some combination of them.

(*a*) additional lattice points in the cell,
(*b*) molecular association.

Considering first (a), it is not difficult to see that the elimination of centring by the above data also eliminates the possibility of any additional lattice points except in the (*ac*) net, and that if, as may safely be assumed, the *a*- and *c*-translations are undivided, the only possibility is trisection of [101] or of $[10\bar{1}]$ by additional lattice points. This would be revealed by the absence of planes $\{hkl\}$ for which $(h + l)$ or $(h - l)$ is indivisible by 3. On the two oscillation photographs mentioned above there were indexed, however, 45 planes of the former and 54 planes of the latter kind, so that clearly the axes as chosen define a primitive monoclinic cell, lattice T_m.

On a series of oscillation photographs about [010], some 23 planes of type $\{h0l\}$ had an opportunity to be recorded. Of the 10 which produced spots, 5 possessed index h odd, 5 index l odd, and none $(h + l)$ odd, while of some 13 absentees, all except (301) possessed $(h + l)$ odd. Hence it may safely be concluded that the space group possesses glide plane translations parallel to [101] and [10$\bar{1}$].

From two more series of oscillation photographs the following data were obtained with regard to reflections from $\{0k0\}$:

k = 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, present,
k = 3, 7, 9, 11, 13, 15, 17, 19, 21, 25, absent,
k = 1, 5, probably absent,
k = 24, uncertain.

The space group therefore possesses a digonal screw axis parallel to the b-axis.

Only those monoclinic space groups which are based on the lattice Γ_m are precluded by observed reflections in this case; but if, as seems very likely, the following are true space group halvings:

$\{h0l\}$ if $(h + l)$ is odd,
$\{0k0\}$ k ,

then the correct space group must be C_{2h}^5 $(C_{2h}pv2)$. If we choose origin at the intersection of a glide plane with a digonal screw axis (Astbury and Yardley, Fig. 14), the symmetry elements of the cell may be described as follows:

Centres of symmetry at the intersections of the planes:

$$x = \tfrac{1}{4}, \tfrac{3}{4};\ y = \tfrac{1}{4}, \tfrac{3}{4};\ z = \tfrac{1}{4}, \tfrac{3}{4}.$$

Glide planes: $y = 0, \tfrac{1}{2}$; translations $\tau_x \pm \tau_z$.

Digonal screw axes: intersections of planes:

$$x = 0, \tfrac{1}{2};\ z = 0, \tfrac{1}{2}.$$

As there are only two molecules in the unit cell, they will possess centres of symmetry, which may be at any of the following pairs of centres:

$(\tfrac{1}{4}, \tfrac{1}{4}, \tfrac{1}{4})$ and $(\tfrac{3}{4}, \tfrac{3}{4}, \tfrac{3}{4})$
$(\tfrac{1}{4}, \tfrac{1}{4}, \tfrac{3}{4})$ $(\tfrac{3}{4}, \tfrac{3}{4}, \tfrac{1}{4})$
$(\tfrac{1}{4}, \tfrac{3}{4}, \tfrac{3}{4})$ $(\tfrac{3}{4}, \tfrac{1}{4}, \tfrac{1}{4})$
$(\tfrac{1}{4}, \tfrac{3}{4}, \tfrac{1}{4})$ $(\tfrac{3}{4}, \tfrac{1}{4}, \tfrac{3}{4})$,

which are of course physically equivalent, since a simple translation of the cell axes will transform any one pair into any other pair.

β-Cyclohexandiacetate 1.4

No crystallographic data on this substance have been found in the literature. Good crystals were obtained from 95% alcohol, and examination yielded the following information:

Crystal class: monoclinic prismatic.

$a : b : c = 2.344 : 1 : 1.168.$
$\beta = 107.4°.$

Observed forms: a {100}, c {001}, q {201}, r {210}.
Tablets {100}.

A rough determination of the density gave the value 1.18 gm. cm.$^{-3}$.

Both Laue and oscillation photographs showed the symmetry to be expected from monoclinic crystals. Adopting the same axial system as that used for the goniometric examination, the following results were obtained:

a = 13.56 Å   Cell volume = 507 Å^3
b = 5.83 Å
c = 6.72 Å $C_{10}H_{14}O_4$ per cell = 2
$a : b : c$ = 2.33 : 1 : 1.153   Density = 1.146 gm. cm.$^{-3}$.

From the indexing of one oscillation photograph obtained with a cylindrical camera, it was found that reflections occur from the following groups of planes {hkl}:

28 with $(k + l)$ odd,
27 $(l + h)$,
20 $(h + k)$,
24 $(h + k + l)$.

The lattice is therefore simple monoclinic Γ_m.

For the space group determination it was found from six photographs by rotation about [010] that the following groups of planes of type {$h0l$} reflect:

0 with h odd,
6 l ,
6 $(h + l)$,

while, of the absentees, 9 out of 11 possessed index h odd. From a series of six oscillation about the normal to (100) the following information was obtained with regard to the reflections from $\{0k0\}$:

$$k = 2, 4, 6, \text{ present,}$$
$$k = 1, 3, 5, 7, \text{ absent.}$$

As in the case of β-cyclohexandiol, observed reflections preclude only those space groups based on the centred monoclinic lattice. If, however, as is indicated by the above data,

$$\{h0l\} \text{ if } h \text{ is odd,}$$
$$\{0k0\} \text{ if } k \text{ is odd,}$$

are true space group halvings, then the only possible space group is $C_{2h}^5(C_{2h}pa2)$. In this case, however, the axes are orientated differently, and if the origin is chosen as before, the symmetry elements must be described as follows:

Centres of symmetry at the intersections of the planes:

$$x = \tfrac{1}{4}, \tfrac{3}{4};\ y = \tfrac{1}{4}, \tfrac{3}{4};\ z = 0, \tfrac{1}{2}.$$

Glide planes: $y = 0, \tfrac{1}{2}$; translation τ_x.

Digonal screw axes: intersections of the plane:

$$x = 0, \tfrac{1}{2};\ z = 0, \tfrac{1}{2}.$$

The co-ordinates of the four equivalent points are in this case:

$$x, y, z;\ x + \tfrac{1}{2}, \bar{y}, z;\ \bar{x}, y + \tfrac{1}{2}, \bar{z};\ \tfrac{1}{2} - x, \tfrac{1}{2} - y, \bar{z};$$

and as there are only two molecules in the unit cell, they must be centro-symmetric with their centres at any of the four physically equivalent pairs of positions:

$(\tfrac{1}{4}, \tfrac{1}{4}, 0)$	and	$(\tfrac{3}{4}, \tfrac{3}{4}, 0)$
$(\tfrac{1}{4}, \tfrac{3}{4}, 0)$		$(\tfrac{3}{4}, \tfrac{1}{4}, 0)$
$(\tfrac{1}{4}, \tfrac{1}{4}, \tfrac{1}{2})$		$(\tfrac{3}{4}, \tfrac{3}{4}, \tfrac{1}{2})$
$(\tfrac{1}{4}, \tfrac{3}{4}, \tfrac{1}{2})$		$(\tfrac{3}{4}, \tfrac{1}{4}, \tfrac{1}{2})$.

SUMMARY AND DISCUSSION

The accompanying table summarizes the data which have been obtained on the four substances α- and γ-cyclohexandiol 1.2, β-cyclohexandiol 1.4 and β-cyclohexandiacetate 1.4. The four headings refer by obvious abbrevations to the four substances. The tenth row (Arrangement of Space Group) gives enough data to determine the arrangement of the symmetry elements of the space group with respect to the crystallographic axes. The remark "molecules asymmetric" in the last row of course simply means that the atoms are not so arranged in the crystal that the molecules possess any element of symmetry in common with the space group.

	α-diol 1.2	γ-diol 1.2	β-diol 1.4	β-diacetate 1.4
Formula	$C_6H_{10}(OH)_2$	$C_6H_{10}(OH)_2$	$C_6H_{10}(OH)_2$	$C_6H_{10}(CH_2CO_2)_2$
Melting Point	100°	104°	139°	102°
Density (gm.cm.$^{-3}$)	1.20	1.16	1.19	1.15
a Å	7.62	19.13	6.32	13.56
b Å	8.55	9.92	21.2	5.83
c Å	19.57	7.23	7.27	6.72
β	—	103.9°	96.0°	107.4°
Cell Volume Å	1280	1332	968	507
Probable Space Group	V_h^{15} $D_{2h}p\gamma\alpha\beta$	C_{2h}^6 $C_{2h}b\alpha 1$	C_{2h}^5 $C_{2h}p\nu 2$	C_{2h}^5 $C_{2h}p\alpha 2$
Arrangement of Space Group	glide planes $(100)\tau_z$ $(010)\tau_x$ $(001)\tau_y$	body centred	glide trans. $\tau_x \pm \tau_z$	glide trans. τ_x
Molecules per cell	8	8	6	2
Remarks	molecules asymmetric	molecules asymmetric	centro-asymmetric $(C_6H_{12}O_2)_2$ groups	centro-symmetric molecules

As in the case of the group of crystals described in the preceding papers (*l*-inositol, *i*-inositol, quercitol and quebrachitol) the melting points and densities of the members of this group do not differ much, though both properties are numerically considerably less than for the higher substituted derivatives. From this one might be tempted to suppose that the disubstitution derivatives possess a more open structure and a weaker intermolecular attraction. And in fact this can be to some extent supported by rough calculations of molecular volumes, using information provided by a recent critical review1) of available organic crystal structure data, but unfortunately the margin of error is just great enough so that nothing certain can be concluded at present. It is hoped shortly to discuss various aspects of this problem in some detail in a separate paper.

An interchange of the *a*- and *c*-axes of the γ-cyclohexandiol suggests that there may be similarity in the arrangement of the molecules in this substance and the α-compound, but at present the comparison must be regarded as merely speculative. The most interesting results are those obtained for the two β-1.4-crystals. Both molecules are centrosymmetric, but in the former case the crystallographic molecule is built of three $C_6H_{12}O_2$-groups, and this as a whole possesses the symmetry centre. Hence only the central $C_6H_{12}O_2$-group must possess a centre of symmetry, while the outer pair may be asymmetric provided that they are properly related one to another. The unusual length of the *b*-axis suggests that the molecules are piled up in this direction, and it has been possible to find an approximate agreement of calculated with observed relative intensities for a fairly simple arrangement, but as the structure in other directions still remains uncertain, results are withheld till they can be presented in more complete form.

The molecules of the β-diacetate might be expected to be fairly lengthy, but the large *c*-dimension cannot be correlated immediately with this guess, since the *c*-axis is the direction of the glide translation. Halving this dimension leaves nothing to choose between the axes as far as indicating anything about the orientation of the molecule is concerned. It should, however, be noted that the *b*-axis is distinctly

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1) S. B. Hendricks, Chem. Rev. No. 4. 1930.

shorter than any axis of any of the compounds investigated in this series, and it may well be hoped that this will provide a restriction which will in some degree simplify the more detailed analysis.

In concluding the writer wishes to acknowledge much assistance from Dr. A. L. Patterson during the course of investigation.

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CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

IV. THE SYNTHESIS OF THE *p*-AMINOBENZYL ETHER OF THE SOLUBLE SPECIFIC SUBSTANCE OF TYPE III PNEUMOCOCCUS AND ITS COUPLING WITH PROTEIN

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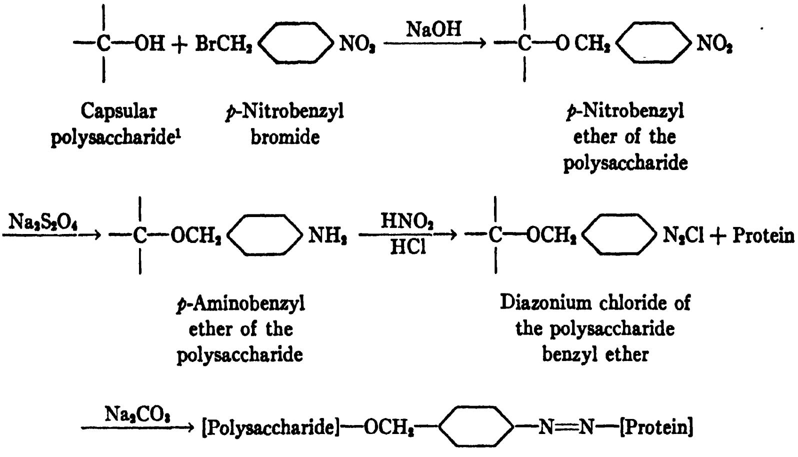
The immunological rôle of specific polysaccharides from encapsulated microorganisms has been discussed in previous communications from this laboratory (1). The capsular carbohydrate of Type III Pneumococcus may be regarded as a pure chemical entity, a polysaccharide free from nitrogenous impurities, retaining the immunological property of reacting specifically in high dilutions with homologous antiserum, yet incapable of inciting the formation of type-specific immune bodies when injected into rabbits. It has been shown, however, that simple carbohydrates when combined with proteins (2) can give rise to specific antibodies. The specificity of the antibodies thus induced is dependent upon the chemical constitution of the carbohydrate irrespective of the protein to which it is bound.

It occurred to us that if the pneumococcus polysaccharide could be combined with a foreign protein it should be possible to produce a conjoined carbohydrate-protein antigen capable of stimulating the formation of type-specific pneumococcus antibodies in the animal body. Provided the specificity of the original carbohydrate has not been too greatly altered either through chemical manipulation, or through the introduction of new molecular groupings, one should obtain on immunization with such a "synthetic antigen," antibodies which would be identical in specific action with those produced by immunization with the intact bacterial cells.

The capsular polysaccharide of Type III Pneumococcus has been shown to be a polymer of an aldobionic acid (3). The carbohydrate itself has an acid equivalent of 338, *i.e.* it has one free carboxyl (COOH) group for every two sugar molecules. In addition to the free carboxyl group there are three free hydroxyl groups (OH) per unit of aldobionic acid in the polymeric form. If the hydrogen atom of one of these three hydroxyl groups can be replaced by a nitrobenzyl group without incurring a loss in specificity of the polysaccharide, the nitro derivative could be reduced to the amino compound which in turn might be coupled through its diazonium derivative to a protein, yielding a conjoined carbohydrate-protein. Such a complex would have only one constituent common to the pneumococcus cell, namely, the capsular polysaccharide.

The synthesis may be diagrammatically represented by the following series of reactions:



Capsular polysaccharide[1]

p-Nitrobenzyl bromide

p-Nitrobenzyl ether of the polysaccharide

p-Aminobenzyl ether of the polysaccharide

Diazonium chloride of the polysaccharide benzyl ether

Conjoined carbohydrate-protein

In the following account a description is given of the experimental procedure used in the chemical synthesis of the *p*-nitro and *p*-aminobenzyl ethers of the specific carbohydrate of Type III Pneumococcus and the coupling of the amino derivative to serum globulin. The immunological specificity of this conjoined carbohydrate-protein complex is discussed in an accompanying publication.

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[1] Since it is impossible to draw a complete formula for the capsular polysaccharide, the latter has been represented by drawing the one carbon atom bearing a free hydroxyl group, which enters into chemical reaction with *p*-nitrobenzyl bromide in the presence of sodium hydroxide.

EXPERIMENTAL

1. Preparation of the p-Nitrobenzyl Ether of Pneumococcus Type III Soluble Specific Substance

The nitrobenzyl ether of the carbohydrate was prepared by a method similar to that employed by Gomberg (4) in the preparation of the benzyl ethers of various carbohydrate derivatives.

1 gm. of the nitrogen-free Type III pneumococcus polysaccharide was suspended in 35 cc. of water and brought into solution by neutralizing with N/1 sodium hydroxide. To the solution was added 4.7 gm. of finely pulverized *p*-nitrobenzyl bromide. The mixture was heated to 100°C. and vigorously stirred. The nitrobenzyl bromide melted to form an oil which could be fairly well emulsified in the solution by violent stirring. 2.9 cc. of 30 per cent sodium hydroxide were added drop by drop. The rate of addition of the alkali was such that at no time were there more than a few drops in excess. The reaction was complete after ¾ hour of heating and stirring, *i.e.*, the theoretical quantity of alkali had been added to neutralize the hydrobromic acid from the *p*-nitrobenzyl bromide, leaving a neutral solution. The contents of the tube was now distilled with steam to rid the reaction mixture of *p*-nitrobenzyl alcohol. The product remaining in the flask, a yellow paste, was cooled to 0°C. and was acidified by the addition of hydrochloric acid. The product was filtered on a hardened paper, washed with small portions of ice water, and finally was dried in a vacuum desiccator.

The dry material was pulverized in a mortar and extracted with acetone in a Soxhlet extractor. A quantity of soluble colored material,—probably condensation products from the *p*-nitrobenzyl bromide, was thus eliminated. The polysaccharide-ether remaining in the extraction thimble was dissolved in alkali, made up to 200 cc. with water, cooled to 0°C., and acidified by the addition of hydrochloric acid. The precipitate (the nitrobenzyl ether of the soluble specific substance) was centrifuged and then reprecipitated. The final product was filtered on a hardened paper and was then dried in a desiccator. About 1.1 gm. of material were recovered.

The nitrobenzyl ether of the soluble specific substance of Pneumococcus was thus obtained as a pale yellow compound, soluble in dilute alkali by virtue of the free carboxyl group in the molecule. The compound itself is insoluble in water, though its sodium salt is readily soluble. The compound may be precipitated by adding acid to an aqueous solution of its sodium salt. The ether does not dissolve in the usual organic solvents. It is soluble in 75 per cent aqueous acetone, and is slightly soluble in 50 per cent alcohol.

The compound has a specific optical rotation of −26.50°, an acid equivalent of 480 (the calculated value for a mononitrobenzyl ether of a polymer of the aldobionic acid $C_{11}H_{19}O_{10}COOH$ = 473), and a nitrogen content of 2.99 per cent (calculated value = 2.96 per cent). The substance reacts specifically with Type III antipneumococcus serum in dilutions of 1:5,000,000.

2. *Preparation of the Aminobenzyl Ether of the Soluble Specific Substance of Type III Pneumococcus*

1 gm. of the nitrobenzyl ether of the soluble substance was suspended in 20 cc. of water. The substance was brought into solution by neutralization with 20 per cent sodium hydroxide. The solution was warmed to 50°C. and to it was slowly added a freshly prepared saturated solution of sodium hydrosulfite. The solution was kept near the neutral point after each addition of hydrosulfite, by the cautious addition of alkali. When a slight excess of the reducing agent persisted, as determined by the ability of the reaction mixture to bleach litmus paper, the solution was cooled in a freezing mixture and then carefully acidified with hydrochloric acid (sp. gr. 1.09). A yellow precipitate of the amino polysaccharide-ether separated out. After centrifugation, it was dissolved, and the solution was dialyzed against successive changes of distilled water until free of chlorides and sulfates. The solution was then poured into 25 volumes of chilled acetone, and after it had flocked out of solution, the amino ether was filtered on a hardened paper. About 0.8 gm. was recovered.

The aminobenzyl ether of the Type III soluble specific substance was obtained as a yellow ash-free amorphous powder soluble in warm water, and in aqueous acetone (75 per cent). It has an optical rotation of −28.5°, an acid equivalent of 453 (calculated = 443.2), and a nitrogen content of 3.15 per cent (calculated = 3.16 per cent). The substance reacts with Type III antipneumococcus serum in dilutions of 1:5,000,000. When weighed, dried samples of this amino compound are dissolved in water, and then 2.2 equivalents of hydrochloric acid added, and the solution titrated at 0°C. with N/20 sodium nitrite, it is found that the derivative uses up exactly the theoretical quantity (calculated on the nitrogen basis) to form the diazonium derivative. Our first few preparations of the amino ether utilized only some 75 per cent of the theoretical quantity of nitrite when titrated. Later preparations, however, appeared to have all of the nitrogen in the amino form, for they utilized the theoretical quantity of nitrous acid.

3. The Coupling of the Aminobenzyl Ether of the Soluble Specific Substance to Serum Globulin

250 mg. of the aminobenzyl ether soluble specific substance were dissolved in 25 cc. of water. The solution was cooled to 0°C. and to it was added 2.2 mols of normal hydrochloric acid. To the opalescent solution was now added 1 mol of N/10 sodium nitrite. The solution was stirred for 20 minutes. Normal sodium hydroxide was then added *very* cautiously until the solution was just neutral to litmus paper. The solution of diazotized soluble substance was now poured into an alkaline solution of serum globulin, (prepared from normal horse serum by repeated precipitation with half saturation of ammonium sulfate) containing 800 mg. of the latter dissolved in 30 cc. of N/2 sodium carbonate at 0°C. An orange color soon developed which deepened on standing, until finally, after 2 hours standing at 0°C., the presence of free diazonium body could no longer be demonstrated. The deeply colored solution was now acidified carefully by the addition of 10 per cent trichloracetic acid. A yellow precipitate separated, which was centrifuged at low speed. The supernatant liquid still contained relatively large quantities of uncombined protein, and some free soluble substance. It was discarded.

The precipitate was suspended in 30 cc. of salt solution and was briskly stirred for 30 minutes to break up any lumps which were present. As soon as a uniform suspension of the yellow precipitate was obtained, it was dissolved by the addition of N/10 sodium hydroxide. The fine particles swelled and gave what was apparently a solution, but a large amount of transparent orange jelly could be centrifuged away from some of the true solution of the substance. However, instead of centrifuging and discarding this jelly, the entire suspension was again acidified with a small amount of trichloracetic acid. The precipitate thus formed was again centrifuged and the clear supernatant liquid was tested for the presence of specifically reacting polysaccharide. Solution and reprecipitation were repeated until no more reactive polysaccharide could be found in the supernatant liquid. This required, in all, about three precipitations.

The final precipitate was now suspended in 30 cc. of 0.9 per cent salt solution containing 0.25 per cent tricresol; N/10 sodium hydroxide was added until the mixture was very faintly alkaline to litmus, but not alkaline to phenolphthalein. Most of the compound went into solution, a small amount still remained in suspension as a jelly-like conglomerate. The mixture was diluted to 150 cc. with 0.25 per cent tricresol salt solution and was used for immunization purposes.

The conjoined carbohydrate-protein reacted with Type III antipneumococcus serum in dilutions of 1:500,000. When the precipitate thus formed was separated by centrifugation, it was found to be colored yellow, and when it was dissolved in alkali, an orange solution was obtained. This was an indication that the specific polysaccharide actually is bound to the protein by way of the chromophoric linkage —N=N— and that when the specific part of the protein-carbohydrate complex reacts with pneumococcus antibody, the complex precipitates *in toto*. The carbohydrate-protein was found to contain 13 per cent of sugar, calculated as glucose. This is in fair agreement with the amount of bound polysaccharide calculated from the reactivity of the carbohydrate derivative with pneumococcus antiserum, *i.e.* roughly 10 per cent, since the sensitivity of this reaction is 1:5,000,000. This polysaccharide-protein complex has been used to immunize animals. The results of the immunological studies are presented in the following paper.

SUMMARY

1. The *p*-amino and *p*-nitromonobenzyl ethers of the specific polysaccharide of Type III Pneumococcus have been prepared.

2. The diazonium ether of the specific polysaccharide has been coupled with serum globulin to yield a specific polysaccharide-protein complex and this complex has been used for immunization. The results of the immunological studies are presented in the following paper.

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CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

V. The Immunological Specificity of an Antigen Prepared by Combining the Capsular Polysaccharide of Type III Pneumococcus with Foreign Protein

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The fundamental studies of Landsteiner and his coworkers (1) on complex antigens have established the important principle that the introduction into the protein molecule of a simple non-protein radical confers a new immunological specificity on the antigenic compound. Furthermore, this newly acquired specificity has been shown to depend upon the nature of the new chemical grouping thus introduced.

Previous studies from this laboratory (2) on the chemo-immunological properties of "synthetic antigens," prepared by combining a simple carbohydrate radical with protein, have shown that the specificity of the newly formed compounds is determined in each instance by the chemical individuality of the reactive carbohydrate, irrespective of the protein to which it is attached. Simple derivatives of glucose and galactose, which by themselves are non-antigenic will, when coupled to a common protein, stimulate the formation of antibodies that are specific for the particular sugar used. Antisera, produced by immunization with the conjugated sugar-proteins, invariably reflect the controlling influence of the carbohydrate on the specificity of the whole antigen. So sensitive is this chemo-specific effect, that mere differences in the spatial arrangement of the groups on a single carbon atom in two glucosides otherwise identical, were found sufficient to change completely the antigenic specificity of the respective compounds.

In order to test the possibility of synthesizing a specific antigen by combining a bacterial carbohydrate with a foreign protein, the specific capsular polysaccharide of Type III Pneumococcus was purposely chosen, since in its purified form it contains no nitrogen and may be regarded as a definite chemical entity. Further, if results were obtained by the use of this particular bacterial polysaccharide, they would be the more significant, since the isolated pure substance alone has never been found to elicit antibodies in rabbits, and even the intact bacterial cells from which it is derived commonly fail to incite the formation of type-specific antibodies in these animals.

From a chemical point of view, the difficulty lay in synthesizing the appropriate derivative of the capsular polysaccharide. It must be one capable of coupling with protein on diazotization and one in which the chemo-specific groups of the bacterial sugar are not masked or destroyed in its preparation. The details of the chemical methods used in the synthesis of the aminobenzyl ether of the capsular polysaccharide of Type III Pneumococcus have been described in the preceding paper (3). The diazonium derivative of the polysaccharide was coupled with globulin prepared from horse serum, and the newly formed compound was tested for antigenicity by repeated intravenous injection in rabbits. The sera of the immunized animals were tested for the presence of type-specific agglutinins and protective antibodies against encapsulated, virulent strains of Type III Pneumococcus and for precipitins against the type-specific capsular polysaccharide and its aminobenzyl derivative. The treated rabbits were subsequently infected with a rabbit-virulent strain of Type III Pneumococcus to determine whether, as a result of immunization, they had acquired active immunity against Type III infection.

In evaluating the immunological findings presented in this paper, the fact should be borne in mind that the antigen used in the experiments has in common with the Type III Pneumococcus only the specific capsular polysaccharide, and that the protein with which it is conjugated is of widely remote biological origin.

EXPERIMENTAL

Methods

Immunization.—Rabbits were immunized by the intravenous injection of from 1 to 2 cc. of solutions of the conjugated carbohydrate-protein antigen daily for six doses, and the course of injections was repeated a second time after a rest period of 7 days. 8 days after the last injection the rabbits were bled and the serum tested for type-specific pneumococcus antibodies.

Antigen.—The antigen, prepared as described in the preceding paper, was preserved by the addition of 0.25 per cent tricresol. Each preparation was standardized on the basis of nitrogen content, so that 1 cc. of the solution contained 5 mg. of protein. This method, however, does not indicate the amount of bound carbohydrate and hence is not an accurate measure of the effective antigenic complex. The protein, to which the aminobenzyl ether derivative of Type III polysaccharide was bound by the diazo reaction, was globulin prepared from horse serum by precipitation with ammonium sulfate.

Technic of Immunity Reactions.—In the precipitin reactions, the immune serum was diluted in the proportion of two parts of serum to three of salt solution and 0.5 cc. of this dilution containing 0.2 cc. of original serum was added to 0.5 cc. of varying dilutions of reacting substance. The final concentration of precipitinogen in the reaction mixture is shown in the protocols. The protection tests were done by the usual technic; mice were injected intraperitoneally with 0.2 cc. immune serum together with varying dilutions of an actively growing culture of Pneumococcus. The dilutions were so made that the total volume injected was 1 cc. in all instances.

I. Type-Specific Antipneumococcus Antibodies

1. Precipitins.—The sera of rabbits immunized with the Type III "synthetic antigen" were tested for the presence of precipitins for the original capsular polysaccharide and for the aminobenzyl ether derivative used in preparing the antigen. The introduction of the para-aminobenzyl radical into the polysaccharide molecule did not destroy the immunological specificity of the derivative, since the latter reacted with Type III antipneumococcus horse serum in high dilutions, showing a specific reactivity comparable to that of the original polysaccharide from which it was derived. It was of interest, therefore, to determine whether this specific carbohydrate derivative, when bound with protein, would stimulate the formation of precipitins reactive not only with the amino derivative but also with the polysaccharide itself, when both substances were used in the free, soluble form.

The precipitin reactions of immune rabbit serum with the original and modified form of the Type III capsular polysaccharide are compared in Table I. The results show that the serum of a rabbit immunized with the "synthetic antigen" reacts with the native polysaccharide and with its aminobenzyl ether in equally high dilutions.

Similarly, as previously pointed out, the serum of a horse immunized with the intact bacterial cells containing the natural Type III antigen precipitates the polysaccharide derivative as well as it does the polysaccharide itself. The evidence is, therefore, that the specifically reactive groups of the polysaccharide have not been chemically masked in forming the new derivative, and that when this specifically reactive carbohydrate is chemically bound with protein to form a new antigen, *it orients the immune response as specifically as does the polysaccharide itself in the form in which it exists as a natural antigen in the encapsulated cells.*

TABLE I

Precipitins for the Capsular Polysaccharide of Type III Pneumococcus in Serum of Rabbits Immunized with Type III Synthetic Antigen

Dilution of carbohydrate	Purified preparation of Type III polysaccharide	Aminobenzyl ether of Type III polysaccharide
1:100,000	++±	+++
1:200,000	+++	+++
1:400,000	++±	+++
1:800,000	+±	+
1:1,600,000	+	±

+++ = complete precipitation with compact disc.
++ = diffuse turbidity.
+ = slight turbidity.

2. *Agglutinins.*—The sera of rabbits, prepared by serial injections of the chemically combined antigen, specifically agglutinate encapsulated strains of Type III pneumococci.

The results of the agglutination reactions, as illustrated in Table II, show that while the titre of agglutinins is not high, the serum is type-specific in that it agglutinates only Type III pneumococci, and fails to react with organisms of Type I and Type II. The fact that the immune serum does not agglutinate the non-encapsulated R forms of Pneumococcus was to be expected, since the antigen contains none of the somatic constituents commonly present in all pneumococci. The lack of these cellular substances in the artificial antigen is reflected in the serum by the absence of the species-specific antibodies, which are ordinarily present when the whole cell is used as the immunizing agent.

TABLE II

Specificity of Agglutination Reaction with Serum of Rabbit Immunized with Type III Synthetic Antigen

Pneumococcus	Immune serum			
	1:5	1:10	1:20	1:40
Type I	–	–	–	–
Type II	–	–	–	–
Type III	++++	+++	+	±
"R" strain*	–	–	–	–

++++ = complete agglutination with disc formation.

– = no reaction.

* Derived from Pneumococcus Type II.

TABLE III

Protective Action in Mice of Sera of Rabbits Immunized with Type III Synthetic Antigen

Pneumococcus Type III	Immune rabbit sera (0.2 cc.)								Virulence controls No serum	
	(*a*)		(*b*)		(*c*)		(*d*)			
cc.										
0.01	–	–	–	–	D 22	D 22	D 7	D 24	–	
0.001	–	–	–	–	S	S	S	S	–	
0.0001	S	S	S	S	S	S	S	S	–	
0.00001	S	S	S	S	S	S	S	S	–	
0.000001	S	S	S	S	S	S	S	S	D 42	D 42
0.0000001	–	–	–	–	–	–	–	–	D 42	D 45
0.00000001	–	–	–	–	–	–	–	–	D 75	D 45

S = survival.

D = death of animal, the numeral indicates the number of hours elapsing before death.

– = not done.

3. Protective Antibodies.—To ascertain whether passive immunity against pneumococcus infection could be conferred on mice by the injection of the serum of a rabbit immunized with the artificial Type

III antigen, protection tests were carried out by the technic described. A constant amount of immune serum (0.2 cc.) was injected intraperitoneally into mice together with varying quantities of a virulent culture of Pneumococcus.

The results given in Table III show that the immune rabbit sera were effective in protecting mice against 10^{-3} cc. of a virulent culture of Type III Pneumococcus, of which 10^{-8} cc. was invariably fatal when injected alone into untreated control animals. Although the maximum degree of protection afforded by the serum was not high, the special interest of these experiments lies in the fact that the protective antibodies were produced in response to a combined antigen containing a specifically reactive carbohydrate which by itself fails to evoke any immune response in rabbits.

TABLE IV

Type Specificity of Protective Antibodies in the Sera of Rabbits Immunized with Type III Synthetic Antigen

Immune serum	*Amount of culture*	Pneumococcus		
		Type I	Type II	Type III
cc.	*cc.*			
0.2	10^{-3}	–	–	S S
0.2	10^{-4}	D 28 D 28	D 28 D 28	S S
0.2	10^{-5}	D 46 D 28	D 28 D 46	S S
0.2	10^{-6}	D 28 D 46	D 28 D 28	S S
Virulence controls	10^{-6}	D 46	D 28	D 46
	10^{-7}	D 72	D 46	D 46
	10^{-8}	S†	D 46††	D 46†††

S = survival.

D = death of animal; the numeral indicates the number of hours elapsing before death.

– = not done.

† The number of colonies developing in blood agar seeded with this inoculum = 0.

†† The number of colonies developing in blood agar seeded with this inoculum = 3.

††† The number of colonies developing in blood agar seeded with this inoculum = 2.

The specificity of the protective antibodies in the sera of rabbits immunized with the Type III synthetic antigen is shown in Table IV. The protective action of the immune serum is strictly type-specific. Mice receiving the immune serum survived infection with Type III Pneumococcus, but succumbed promptly to minimum doses of Type I or Type II culture. The marked specificity of the protection afforded by the serum is further evidence that the antibodies concerned in the reaction derive their specificity from the polysaccharide component of the whole antigen. The protein constituent of the antigen appears to contribute nothing to the antibacterial properties of the serum, except in so far as it renders the carbohydrate antigenic.

4. *Active Immunity.*—Of the series of rabbits injected at various times with effective preparations of antigen, none have failed to respond with the formation of type-specific antibodies. It was of interest, therefore, to ascertain whether rabbits in the sera of which Type III Pneumococcus antibodies were demonstrable had acquired active immunity against infection with this organism. In order to determine this point, several of the treated animals were subsequently infected with multiple lethal doses of a virulent culture of Type III Pneumococcus.

11 days after the last injection of antigen, four immunized rabbits whose serum contained type-specific antibodies were infected by the intradermal injection of 0.2 cc. of an 18 hour blood broth culture of a rabbit-virulent strain of Type III Pneumococcus. The virulence of this strain, maintained by repeated rabbit passage, was such that 0.00001 cc. of the culture alone injected into the skin proved fatal within 72 hours.

Four rabbits, which had received repeated injections of the "synthetic antigen," were reserved and later tested for active immunity. All four of these rabbits survived infection. In the two animals which had the highest titre of type-specific antibodies in their serum, the infection ran a practically afebrile course with only a slight inflammatory reaction at the point of inoculation; in the other two rabbits, the temperature remained relatively low and the skin lesion was not severe. At no time during the course of the infection were pneumococci present in cultures of the blood of the immunized rabbits, whereas in the normal controls the bacteremia invariably increased up to the time of death.

The results demonstrate that an antigen prepared by combining the Type III capsular polysaccharide with an animal protein is capable of inducing in rabbits an active immunity against infection with a virulent culture of Type III Pneumococcus. The only constituent of Pneumococcus in the artificial antigen is the capsular polysaccharide. Since this carbohydrate alone is non-antigenic in rabbits, the antibacterial immunity induced by the combined antigen can be ascribed only to the antigenicity acquired by the polysaccharide through combination with the foreign protein. The results bring evidence that an effective, active immunity can be developed in which the only antibacterial antibodies formed are those directed against the capsular component of the pneumococcus.

II. Antiprotein Antibodies

In the conjugated antigen, the protein used in combination with the Type III capsular polysaccharide was globulin derived from normal horse serum. The total protein of the antigen was estimated by determining the nitrogen content of the various preparations. This method of standardization, while affording a measure of the total globulin present, does not indicate the amount of protein that is bound with the polysaccharide to form the effective type-specific antigen. The sera of immune rabbits invariably contained not only type-specific antibodies for Type III Pneumococcus, but also specific precipitins for the globulin of horse serum.

The presence in immune rabbit serum of precipitating antibodies for the foreign protein used in preparing the antigen is shown in Table V. The antiprotein precipitins evoked by the combined antigen may be attributed to one or both of two possibilities. In the first place, despite attempts to remove the excess of unbound globulin from solution, it is possible that sufficient protein remains free and uncombined with the polysaccharide to function independently as antigen. It is also possible, however, that even in the absence of any free globulin, the combined antigen itself may give rise to two qualitatively different antibodies, each specifically related to the corresponding component of the antigenic complex. This explanation of the concurrence of two distinct varieties of antibodies in the immune serum involves the assumption, that while the carbohydrate has acquired antigenicity through combination with the globulin, the protein molecule itself has not lost its own specific antigenic property. According to this view, a single compound of carbohydrate and protein may possess the dual antigenic property of stimulating two separate and specific antibodies; one evoked by the newly acquired antigenicity of the polysaccharide in union with globulin, and the other stimulated by the protein molecule itself in which the chemo-specific groups essential for antigenicity have not been masked in the linkage with the carbohydrate.

TABLE V

Antiglobulin Precipitins in Sera of Rabbits Immunized with Type III Synthetic Antigen

Immune sera	Globulin (horse serum)*			
	1:20,000	1:40,000	1:80,000	1:160,000
(a)	+++	+++	++	±
(b)	+++±	+++	++	+
(c)	++++	+++±	+±	±
(d)	++++	+++	+±	+
(e)	+++±	++±	+	±

++++ = flocculent precipitation, clear supernatant.
+ = slight, diffuse turbidity.

* The diazonium derivative of the capsular polysaccharide of Type III Pneumococcus was coupled with globulin from horse serum.

Of the two explanations, the first, namely that the free globulin in the antigenic mixture accounts for the antiprotein antibodies, is the simpler and perhaps the more likely one. However, the second concept, which ascribes a dual antigenicity to the sugar-protein, affords an immunologically interesting and theoretically possible explanation.

DISCUSSION

In the present state of our knowledge it would be hazardous to predict the precise conditions under which complex carbohydrates by themselves may function as antigens. The present study simply defines certain experimental conditions under which the capsular polysaccharide of Type III Pneumococcus, in chemical union with a foreign protein, is rendered specifically antigenic in a particular species of animals, in which the carbohydrate alone has never been found to incite antibody formation. The "synthetic antigen" elicits in rabbits a type-specific antipneumococcus response, which neither one of its constituents alone is capable of inciting when injected singly into these animals.

In order to effect the chemical union, it was first necessary to synthesize a derivative of the bacterial carbohydrate that could be diazotized and coupled to protein by means of the diazo linkage. This was accomplished by forming the aminobenzyl ether of the polysaccharide, a derivative which fulfills the chemical requirements while still retaining the serological specificity of the original carbohydrate.

Antisera prepared by immunization with the combined antigen contain type-specific antibodies which precipitate the Type III capsular polysaccharide, agglutinate pneumococci of the homologous type, and protect mice against infection with virulent strains of Type III Pneumococcus. The fact that, in the present instance, the antipneumococcus response is produced by an antigen known to contain but a single component of the bacterial cell, indicates the unity of the antibodies participating in the type-specific reactions of precipitation, agglutination, and protection, and relates the specificity of these antibodies to that of the capsular polysaccharide in the reactive part of the antigenic molecule.

CONCLUSIONS

1. Type-specific antipneumococcus immunity has been induced in rabbits by immunization with antigen prepared by combining a specific derivative of the capsular polysaccharide of Type III Pneumococcus with globulin from horse serum.

2. Rabbits immunized with this antigen acquire active immunity against infection with virulent Type III pneumococci.

3. The sera of the immune rabbits contain type-specific antibodies which precipitate the Type III capsular polysaccharide, agglutinate Type III pneumococci, and specifically protect mice against Type III infection.

4. The experimental data are discussed with reference to: (1) the concurrence in the immune sera of type-specific antibodies for Pneu-

mococcus and precipitins for horse globulin; (2) the determining influence of the capsular polysaccharide on the specificity of the antigen as a whole; (3) the unity of the type-specific precipitins, agglutinins, and protective antibodies induced by a single component of the pneumococcus in chemical union with an unrelated, animal protein.

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CUTANEOUS REACTIONS IN RABBITS TO THE TYPE-SPECIFIC CAPSULAR POLYSACCHARIDES OF PNEUMOCOCCUS

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It has previously been shown (1) that, following the intradermal injection of the type-specific polysaccharides of Pneumococcus, there develops in the skin of patients convalescent from pneumonia an immediate wheal and erythema reaction to the polysaccharide homologous in type to that of the pneumococcus causing the infection. The reaction is type-specific and its occurrence is always associated with the presence in the patient's serum of specific antibodies for the homologous type of Pneumococcus.

Since the purified carbohydrates are capable of eliciting this striking response in the skin of human individuals who, upon recovery from lobar pneumonia become spontaneously immune, it seemed desirable to study the cutaneous reactivity of experimental animals which had been artificially immunized to type-specific pneumococci. The purpose of this paper, therefore, is to report the occurrence of a local cutaneous reaction to the type-specific polysaccharides in rabbits actively or passively immunized to the pneumococcus.

Materials and Methods

1. *Immunization of Rabbits.*—

(*a*) *Active Immunization.*—Groups of rabbits were immunized with pneumococci of Types I, II or III. Other animals were immunized with a non-type-specific R strain of pneumococcus. Immunization was carried out by the method of Cole and Moore (2). 10 days after the final injection, blood was obtained from an ear vein and the serum tested for the presence of type-specific antibodies.

(*b*) *Passive Immunization.*—Antipneumococcus rabbit serum was obtained from animals immunized as above. Antipneumococcus horse serum for each of Types I, II and III was obtained from the New York State Board of Health. Rabbits were passively immunized by the intravenous injection of 5 to 15 cc. of one or other of these sera. The following day blood was obtained from an ear vein for the determination of circulating type-specific antibodies.

2. Determination of Type-Specific Antibodies.—

(*a*) Type-specific antibodies were detected by the precipitin test; 0.2 cc. of serum diluted to 0.5 cc. with physiological saline was mixed with 0.5 cc. of varying dilutions of the type-specific polysaccharides. Readings were made after 2 hours in the water bath at 37°C. and overnight in the ice box.

(*b*) Type-specific agglutinins were determined by the usual method using dilutions of serum and suspensions of heat-killed organisms.

3. Skin Testing Materials.—Skin tests were made with the capsular polysaccharides of pneumococcus derived in purified form from Pneumococcus Types I, II and III. These substances are chemically and serologically distinct. They are protein-free. The Type II and Type II polysaccharides are also nitrogen-free; the Type I material contains 5 per cent non-protein nitrogen which is part of the sugar molecule (3). The polysaccharides were dissolved in physiological saline in a concentration of 1:200 and 0.1 cc. of this solution, containing 0.5 mg. of polysaccharide, was used for each injection. The animals were closely observed for the occurrence of an immediate or delayed reaction. Measurements were recorded 24 hours following the injection and daily thereafter.

In most instances the specific capsular polysaccharides of Types I, II and III Pneumococcus were injected simultaneously into neighboring sites in the rabbit's skin.

Skin Reactions in Actively Immunized Rabbits

Three series of rabbits were actively immunized with pneumococci of Types I, II and III, respectively. After immunization, injections of the type-specific polysaccharides into the skin were made.

Protocols of rabbits representative of the three different immune groups illustrate the conditions of experiment and characteristic results.

Rabbit 4-74 was actively immunized to Type I Pneumococcus by the intravenous route. 10 days after the final immunizing injection, the animal's serum contained specific precipitins for the Type I polysaccharide in a titer of 2,560,000. The following day injections of 0.5 mg. of each of Types I, II and III polysaccharides were made into the shaved skin of the side. 2½ hours later a few faint purpuric spots were observed at the site of injection of the Type I material. The lesion increased in size until at 24 hours a purpuric edematous area 2.5 cm. in diameter was noted. In 48 hours the diameter had reached 3.0 cm., the edema was less but the purpura had darkened and infiltration had increased. A gradual clearing took place over the next 4 days. At the sites of injection of the Type II and Type III materials no evidence of reaction was observed.

Rabbit 4-41 was actively immunized against Type II Pneumococcus. 10 days after immunization was completed the serum contained precipitins for the Type II polysaccharide in a titer of 1,280,000. The following day, injections of 0.5 mg. of each of the Type I, II and III polysaccharides were made into the skin of the side. 24 hours later, at the site of injection of the Type II polysaccharide, a firm edematous lesion, bright red in color, and measuring 4.5 cm. x 2.3 cm. in cross diameters was noted. No purpura was present. The lesion had practically cleared in 4 days. No reaction was elicited by the carbohydrates of Types I and III.

Rabbit 14-85 was immunized with a strain of Type III Pneumococcus which had, by repeated animal passage, acquired virulence for rabbits. On the 10th day after the last immunizing dose the serum contained precipitins for the Type III polysaccharide. With a 1:80,000 dilution of the polysaccharide a ++++ reaction was obtained. Higher dilutions were not tested. The following day 0.5 mg. of each of the polysaccharides of Types I, II and III was injected into the shaved skin of the side. After 24 hours at the site of injection of the Type III polysaccharide, there was noted a slightly elevated, firm, purpuric area 2.6 x 3.4 cm. in diameter, surrounded by a white zone of edema. The lesion gradually subsided until the 6th day when only a faint pigmentation remained. The reaction was type-specific; no reaction was produced by the Types I and II carbohydrates.

TABLE I

Skin Reactions to Type-Specific Polysaccharides in Rabbits Actively Immunized with Pneumococci of Homologous Types

Type of Pneumococcus used for immunization	Type-specific precipitins present		No type-specific precipitins present	
	No. of rabbits	No. giving skin reaction	No. of rabbits	No. giving skin reaction
I	11	10	4	0
II	9	8	3	0
III	5	3	8	0
R	—	—	5	0

The foregoing protocols demonstrate clearly the form of reaction obtained and the conditions under which it was produced. Table I shows the frequency with which skin reactions were elicited by the intradermal injection of the homologous polysaccharide in rabbits actively immunized with pneumococci of Types I, II or III, or with an R strain of pneumococcus.

It is seen in Table I that of twenty-five animals yielding serum in which type-specific precipitins were present, all but four gave a typical local inflammatory reaction to the injection of the carbohydrate of the homologous type. In these four instances the titer of antibodies was lower than in the reactive animals. On the other hand, those animals in which circulating type-specific precipitins could not be demonstrated failed in every instance to give a positive response. No reaction was obtained in any case except to the homologous type-specific polysaccharide.

In contrast to the immediate wheal and erythema reaction produced by the carbohydrates in patients convalescent from lobar pneumonia, the reaction produced in the artificially immunized rabbits is usually first detected after 6 to 8 hours. The reaction makes its appearance as a faint blush and gradually increases in size, producing a pink edematous area frequently surrounded by a zone of pale edema. The edema tends to increase and erythema is replaced by a purplish discoloration. The lesion reaches its height in 24 to 48 hours and at that time it may be 4 to 5 cm. in diameter and 0.5 to 1.0 cm. in elevation. A gradual regression takes place in 4 to 7 days until little or no trace of the reaction remains. With the doses employed necrosis of the skin has not been observed. The cutaneous reaction was produced in rabbits only by the polysaccharide homologous in type to that of the pneumococcus employed in the process of immunization. The severity of reaction appears to be related to the titer of type specific precipitins for the homologous polysaccharide in the serum of the animal. In certain instances, despite the presence of type-specific precipitins, no cutaneous response was evoked by the carbohydrate, but in no case was a reaction produced in the absence of circulating type-specific precipitins.

Skin Reactions in Passively Immunized Rabbits

Attempts were made to transfer reactivity, passively, to normal rabbits by the intravenous injection of serum from immune rabbits known to give positive skin tests and the serum of which contained a high titer of type-specific precipitins. 5 to 40 cc. of serum were injected. In none of the recipient animals, however, was a positive skin reaction induced, nor were precipitins demonstrable in their sera.

The failure in these instances may be due to the fact that insufficient immune serum was injected.

Attempts were next made to induce skin reactions to the type-specific polysaccharides in animals passively immunized with serum of horses immune to Pneumococcus Types I, II or III. Fourteen rabbits were injected: five with Type I serum, three with Type II serum and six with Type III serum. 10 to 15 cc. of immune serum were injected intravenously, although in one rabbit only 5 cc. of serum were used. The following day the serum of all the passively immunized rabbits was found to contain a high titer of precipitins for the homologous polysaccharide and in each instance a well marked cutaneous response to the intradermal injection of the polysaccharide occurred. The character of the reaction was identical in every respect with that obtained in actively immunized animals. No reactions to the polysaccharides of heterologous types were observed.

Local passive transfer of sensitivity was carried out in rabbits by infiltrating each of two areas of skin with 1.0 cc. of immune horse serum. After 24 hours there were seen at the sites of serum infiltration small flat areas of erythema. Into one of these areas 0.5 mg. of the homologous polysaccharide was injected and into the other, as control, an equal amount of a heterologous polysaccharide. 24 hours later, at the site of inoculation of the homologous polysaccharide, a deep red, elevated edematous area 2 to 4 cm. in diameter was observed and this persisted for 48 to 72 hours. At the control site there remained only a pale erythema due to infiltration of the serum.

Guinea pigs were passively immunized by intraperitoneal injections of antipneumococcus horse or rabbit serum. The animals which received immune horse serum showed no signs of local or general reaction to the subsequent intradermal injection of the homologous type-specific polysaccharide. Similarly, no cutaneous reaction to the polysaccharide was observed in those guinea pigs passively immunized with rabbit serum. In several of the latter group, however, mild signs of general anaphylaxis (restlessness, coughing, scratching of nose, etc.) were noted shortly after the introduction of the specific substance into the skin. Tillett and Avery (4) were able to induce fatal anaphylactic shock with minute amounts of the type-specific polysaccharide in guinea pigs passively sensitized with homologous antipneumococcus rabbit serum. If horse serum was employed for sensitization no response occurred. In the present instances the observation that anaphylactic symptoms were produced in guinea pigs receiving antipneumococcus rabbit serum but not in those prepared with immune horse serum, appears to be another demonstration of the facts reported by these workers.

DISCUSSION

The results reported in the present paper demonstrate that in rabbits actively or passively immunized to a type-specific pneumococcus the intradermal injection of specific polysaccharide of homologous type elicits a definite form of cutaneous reaction. In contrast to the immediate wheal and erythema reaction observed in humans convalescent from pneumonia, the reaction produced in the immune rabbit is delayed and resembles in its general appearance the form of reaction first described by Arthus (5). The reaction is characterized by slow development, edema, erythema and purpuric discoloration. The capacity of the individual rabbit to give a positive skin reaction is closely associated with the presence of demonstrable type-specific precipitins in its serum. In no instance was a positive reaction observed in the absence of demonstrable type-specific precipitins. In the guinea pig no local skin reaction was elicited but signs of general anaphylaxis were observed.

It is plain that one can correlate the reactions induced in immunized individuals of three species (man, rabbit and guinea pig) by the intradermal injection of a protein-free carbohydrate of bacterial origin. The result is the same, apparently, whether the immunity has been actively or passively acquired. In all three species the form of response to carbohydrate is typical of the reactions commonly considered to be of an anaphylactic nature when produced by protein materials.

The reactions are elicited only in the presence of specific antibodies for the capsular polysaccharide of Pneumococcus and then only by the specific polysaccharide homologous in type to that of the pneumococcus inducing the antibody response. Julianelle has shown (6) that animals which have received repeated intravenous injections of unencapsulated R forms of Pneumococcus, or of the nucleoprotein

TABLE II

Relation of Hypersensitiveness and Immunity to Pneumococcus in Rabbits

Material used for immunization	Route of immunization	Antibodies produced		Immunity against virulent pneumococci		Skin reaction to pneumococcus protein	Skin reaction to capsular polysaccharide	Summary
		Species-specific	Type-specific	Active resistance	Ability of serum to protect mice			
Pneumococcus nucleoprotein	i. v. or i. c.	+	−	−	−	+	−	Hypersensitiveness to pneumococcus protein but no immunity to pneumococcus infection
R form of pneumococcus	i. v. or i. c.	+	−	+	−	+	−	Broad increased active resistance to all types of Pneumococcus with protein hypersensitiveness
S Pneumococcus	i. c.	+	Present in low titer in only 12%	+	Protection against high dilutions in only 21%	+	−	Same as above
S Pneumococcus	i. v.	+	+	+	+	+	+	Broad active anti-R resistance. Anti-S type-specific immunity. Hypersensitiveness to protein. Reaction to polysaccharide

of the pneumococcus, or have received intracutaneous injections of unencapsulated or even of encapsulated organisms develop only species-specific, antisomatic antibodies and a hypersensitiveness for the proteins of the pneumococcus. Type-specific antibodies for the capsular polysaccharide could not be detected in the serum of animals so treated, nor were any skin reactions produced by the injection of type-specific polysaccharides. Similarly, in the present experiments no reactions to the carbohydrates were obtained in animals immunized to the R organisms nor in fact, in any animal the serum of which contained no type-specific precipitins. Furthermore, in man, the reaction obtained with nucleoprotein does not take the form of a wheal and erythema, but is a delayed reaction reaching its height in 18 to 24 hours. It is evident, therefore, that the species-specific antisomatic, or antiprotein, antibodies play no rôle in the production of the skin reactions to the capsular polysaccharides. Moreover, the data presented stress again the sharp type specificity of all reactions involving the pneumococcus capsular polysaccharides.

The purpose of this report is not to enter into a discussion of the relation between hypsersensitiveness and immunity but a few noteworthy facts may be mentioned. These are presented in tabular form in Table II. Hypersensitivity to the protein of the pneumococcus can be induced in rabbits by repeated injections of nucleoprotein. Under these conditions, antibodies for the species-specific R Pneumococcus develop but neither active immunity nor any capacity of the serum to confer passive immunity upon another species can be demonstrated (7, 8). In this case, hypersensitivity to the bacterial protein is not associated with increased resistance to pneumococcus infection.

If an R form of Pneumococcus is employed for immunization, only species-specific (anti-R) antibodies appear in the serum of the animal. Protein hypersensitivity can be demonstrated in this animal but no reaction is elicited by any of the capsular polysaccharides (6). In addition, the animal can be shown to have acquired an increased resistance to virulent pneumococci of Types I, II and III (8). Hence, this resistance is not type-specific. The height of the immunity is not great and is passively transferable only with great difficulty within the same species. The serum of such an animal does not confer passive protection upon mice. Here, then, is an example of protein hypersensitiveness associated with a broad, non-type-specific increased active resistance.

When an animal has been effectively immunized with a virulent, encapsulated type-specific pneumococcus, type-specific anticapsular antibodies appear in the circulating blood. The animal has developed not only a highly efficient type-specific active immunity but its serum affords passive protection to mice against many lethal doses of pneumococci of the same type. This type-specific immunity is the form observed in convalescents from lobar pneumonia. In association with immunity due to type-specific antibodies, the intradermal injection of the type-specific capsular polysaccharide incites a response characteristic for the species. The reaction to the intradermal injection of the capsular polysaccharides of Pneumococcus may be termed, therefore, a hypersensitive or anaphylactic response occurring only in the presence of type-specific immunity.

SUMMARY

The injection of the type-specific capsular polysaccharides of Pneumococcus Types I, II and III into the skin of rabbits, actively or passively immunized to one of these types of Pneumococcus, elicits a type-specific cutaneous reaction. The form of reaction resembles that described by Arthus. The reaction is produced only when type-specific precipitins for the homologous polysaccharide are demonstrable in the blood of the rabbit. In 84 per cent of actively immunized rabbits, the serum of which contained type-specific precipitins, a reaction was elicited. A positive result was obtained in 100 per cent of rabbits passively immunized with antipneumococcus horse serum whereas, attempts passively to transfer reactivity from immune rabbit to normal rabbit were unsuccessful. The recipients, in the latter group, possessed no demonstrable circulating type-specific precipitins. The reaction produced by specific capsular carbohydrates is always associated with a well grounded type-specific immunity.

A brief summary of the relation of hypersensitiveness and immunity to pneumococcus is given.

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DURATION OF DEMONSTRABLE ANTIBODIES IN THE SERUM OF RABBITS IMMUNIZED WITH HEAT-KILLED TYPE I PNEUMOCOCCI

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In a previous paper[1] have been reported the results obtained, as regards the development of agglutinating and protective antibodies in the blood, when rabbits are immunized by intravenous, intraperitoneal, intramuscular or subcutaneous injections of heat-killed Type I pneumococci. The duration of time that these antibodies persist in the blood was not discussed, however. In the present study rabbits immunized by these various methods have been studied over long periods of time, until the antibodies have disappeared from the blood or until the animals have died from extraneous causes.

The methods employed were identical with those used in the previous study. The animals were bled 10 days after the last injection and thereafter every 30 days. The immunity response and the persistence of immune bodies in the blood in the rabbits of the several groups are shown in Text-figs. 1 to 4.

Rabbits Injected Intravenously

In Text-fig. 1 is shown the course of development and disappearance of antibodies in the serum of nineteen rabbits which received, during the course of treatment, from 1 to 15 cc. of the suspension of heat-killed organisms. Nine of the rabbits died from various causes before the completion of the observations.

It is evident that agglutinins were demonstrable during only relatively short periods following the immunization. In Rabbit A-14, which received 12 cc. of the bacterial suspension, the agglutination titre of the serum after 10 days was 1:500 but after 30 days the titre had decreased to 1:10, and on the 70th day no agglutinating power

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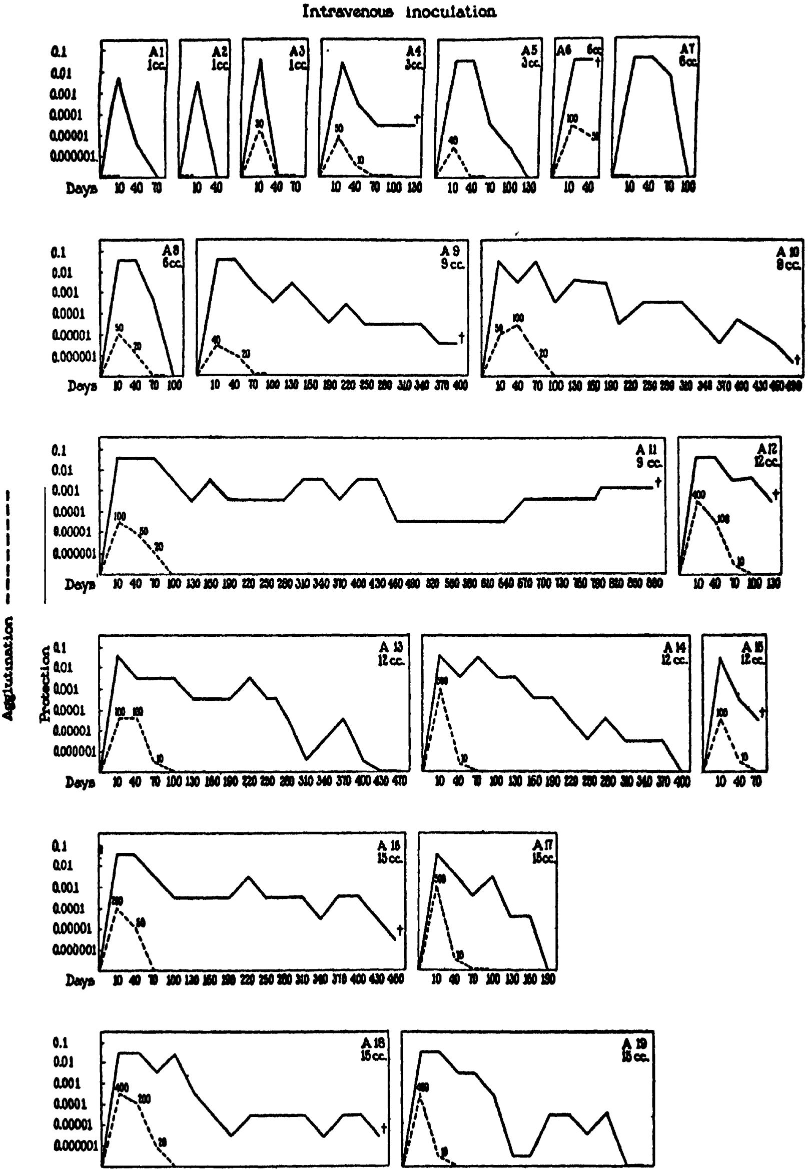
[1] Stillman, E. G., *J. Exp. Med.*, 1930, 51, 721.



TEXT-FIG. 1. Length of time during which agglutinins and protective antibodies could be demonstrated in sera of rabbits which had been immunized intravenously with heat-killed Type I pneumococci.

whatever could be detected. In no instance could type-specific agglutinins be demonstrated longer than 100 days.

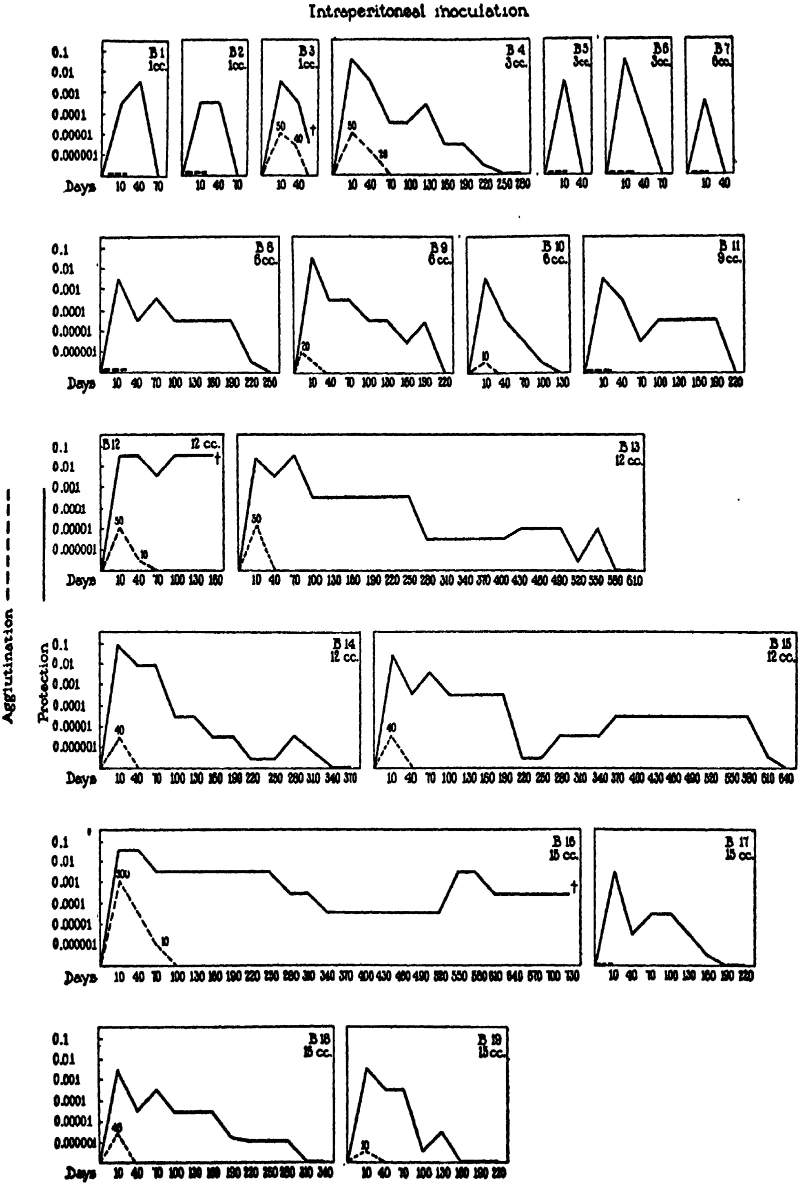
10 days after the course of immunization was ended the sera of all of the animals of this group showed well marked protective power. It is remarkable that the sera of the rabbits which received a total of 1 cc. of the bacterial suspension showed as high a titre of protective antibodies as did the sera of those which received as much as 15 cc. In the animals which received the larger amounts, however, the protective subtances persisted longer than they did in the animals which received smaller amounts. In Rabbit A-11 which received 9 cc. of the suspension, the serum showed high protective power 880 days after the completion of the immunization.

Rabbits Immunized Intraperitoneally

The results in the rabbits immunized by intraperitoneal injections are shown in Text-fig. 2. All but three of these animals lived until demonstrable antibodies had disappeared from the blood. In only ten of the nineteen animals of this series were agglutinins demonstrable and they never reached as high a titre, nor did they persist so long, as in the animals injected intravenously. Protective antibodies were present, however, in the sera of all the animals, and, in the animals which received more than 6 cc. of the suspension, the protective action of the serum was demonstrable for long periods. Sera obtained from two of the rabbits 550 days after the course of immunization was completed still protected mice against 0.0001 cc. of culture, and in the case of one rabbit, serum obtained 730 days after immunization still exhibited protective action.

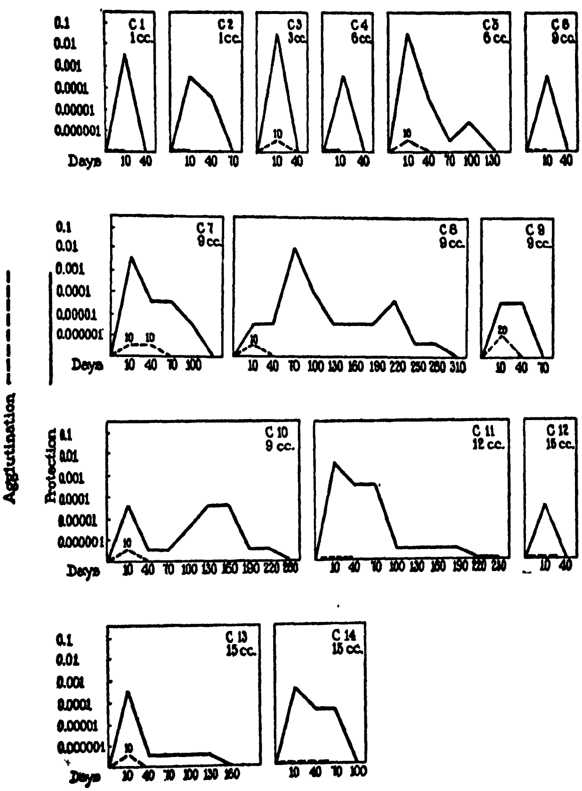
Rabbits Immunized Intramuscularly

Text-fig. 3 shows the number of days during which agglutinins and protective antibodies were detectable in the sera of fourteen rabbits immunized by intramuscular injections. All these animals survived until antibodies could no longer be demonstrated in their blood. The agglutinin titre of the sera from the rabbits of this series was never high and the presence of agglutinins could be demonstrated for only short periods. In fact, in the serum of only one of the rabbits could agglutinins be demonstrated as long as 40 days. The results as re-



TEXT-FIG. 2. Length of time during which agglutinins and protective antibodies could be demonstrated in sera of rabbits which had been immunized intraperitoneally with heat-killed Type I pneumococci.

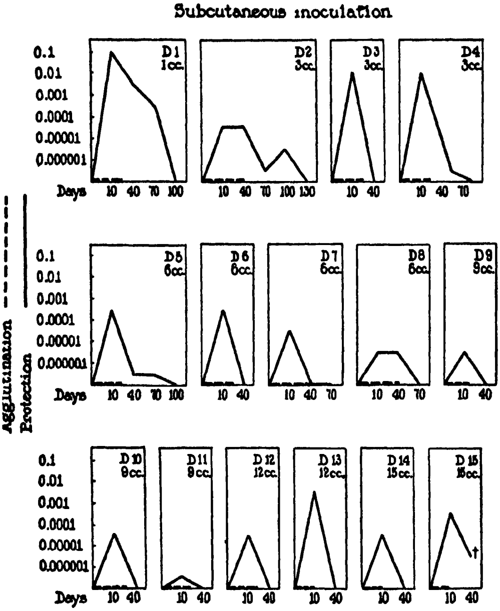
gards the development of protective power were also irregular. Although, in a considerable number of the animals serum obtained 10 days after immunization was highly protective, this property was quickly lost. In only two of the rabbits could protective substances be demonstrated 160 days after inoculation.


Intramuscular inoculation

TEXT-FIG. 3. Length of time during which agglutinins and protective antibodies could be demonstrated in sera of rabbits which had been immunized intramuscularly with heat-killed Type I pneumococci.

Rabbits Immunized Subcutaneously

The results in fifteen rabbits which received subcutaneous injections are shown in Text-fig. 4. In the sera of none of these rabbits were agglutinins demonstrable at any time. Protective antibodies also were present only in low concentrations and they rapidly disappeared. In this series the animals which received the smaller doses not only showed higher concentrations of protective antibodies in the serum than did the animals which received the larger amounts but the antibodies persisted longer.


Subcutaneous inoculation

TEXT-FIG. 4. Length of time during which agglutinins and protective antibodies could be demonstrated in sera of rabbits which had been immunized subcutaneously with heat-killed Type I pneumococci.

DISCUSSION

These experiments show that in rabbits immunized by the injection of heat-killed organisms type-specific agglutinins are demonstrable in the serum only for short periods of time, though the actual length of time that these antibodies persist depends, to some extent, upon the route of immunization employed. In general, the agglutinin titre is highest and the agglutinins persist longest in the animals immunized by intravenous injections; the agglutinins are present in less high titre and they persist for shorter periods in the animals immunized by the intraperitoneal route; they are still less regularly demonstrable and they persist for still shorter periods in the animals immunized by intramuscular injections; while in the animals immunized subcutaneously agglutinins are never demonstrable by the methods we have employed.

It appears that the injection by any route of a suspension of killed pneumococci will stimulate the production of protective antibodies and that when only 1 cc. of the suspension is injected the serum will be of as high a titre as when 15 cc. is injected. The length of time during which protective antibodies persist in the serum, however, varies with the route of injection and the amount of the inoculum. They persist longer in the animals receiving intravenous or intraperitoneal injections than they do in the animals injected intramuscularly or subcutaneously and in general they persist longer in the animals receiving larger amounts of the suspensions than in those injected with smaller amounts.

CONCLUSIONS

In rabbits immunized by the injection of suspensions of heat-killed pneumococci the results obtained as regards not only the development of agglutinating and mouse protective antibodies, but also the persistence of these bodies in the blood depend to a considerable extent upon the route of immunization and the size of the inocula. Agglutinins may appear in the sera of all the rabbits except those inoculated subcutaneously, but in most instances they disappear within a short time.

Protective antibodies appear in the sera of all rabbits, no matter which route of injection be employed and they persist much longer than do the agglutinins. They persist longest when the injections are made intravenously or intraperitoneally and are of briefest duration when the injections are made subcutaneously.

[Reprinted from The Journal of Experimental Medicine, October 1, 1931,
Vol. 54, No. 4, pp. 623–627]

LOCALIZATION OF PNEUMOCOCCI IN THE LUNGS OF PARTIALLY IMMUNIZED MICE FOLLOWING INHALATION OF PNEUMOCOCCI

By ERNEST G. STILLMAN, M.D., and ARNOLD BRANCH, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research*)

(Received for publication, June 3, 1931)

In preceding papers[1] it has been shown that when mice are intoxicated with alcohol and then sprayed with a culture of virulent Type I or Type II pneumococci many of the animals die with a general blood infection, but there rarely occurs any localization of the infection in the lungs. On the other hand, when mice, which have previously been exposed to inhalations of pneumococci of either type, are alcoholized and sprayed, pulmonary localization of the infection with manifest lesions in the lungs often occurs. There are at least two possible explanations of this phenomenon. It is possible that as a result of the first inhalation the tissues of the lungs become sensitized, or altered, so that on the second exposure the infection becomes localized in this organ. The second possibility is that by the first inhalation there is induced a state of general immunity, even of mild grade, and, as is well known, infections in immune animals are likely to be localized at the site of injection. Previous experiments have shown that after repeated inhalations of living virulent Type I pneumococci mice become increasingly resistant to infection with these organisms.

Experiments were undertaken, therefore, to determine whether pulmonary lesions may occur following the inhalation of virulent pneumococci by alcoholized mice that have previously been specifically immunized by routes other than the pulmonary one. In these mice a state of pulmonary sensitization cannot be assumed to be present. Mice were given intraperitoneal injections of (1) normal horse serum (2) Type I or Type II antipneumococcus serum or (3) Type I or Type

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[1] Stillman, E. G., *J. Exp. Med.*, 1924, **40**, 353, 567.

II pneumococcus vaccine, and after 10 days they were alcoholized and exposed to a spray of homologous or heterologous pneumococci.

EXPERIMENTAL

Methods.—Normal horse serum or antipneumococcus horse serum diluted in normal salt solution was injected intraperitoneally into mice in amounts varying from 0.1 cc. to 0.0000000001 cc., the total quantity of fluid injected in each case being 0.5 cc. 3 hours later the animals were alcoholized and exposed to a spray of pneumococcus culture. Other mice were injected intraperitoneally with a suspension of heat-killed pneumococci in doses varying from 0.1 cc. to 0.0000000001 cc. The vaccine was so prepared that 1 cc. of the suspension was equal to 10 cc. of broth culture. 10 days later these mice were intoxicated with alcohol and exposed to a spray of a culture of virulent Type I pneumococci.

All the mice were alcoholized by the intraperitoneal injection of 1.5 cc. of a 10 per cent solution of alcohol in salt solution. 1 hour after the administration of the alcohol the animals were placed in the chamber and sprayed with 50 cc. of broth culture of virulent Pneumococcus Type I. They were exposed in the spray box for 1 hour. In every instance in which fatal infection occurred, cultures were made from the heart's blood, and at least one lobe of the lung was fixed in Zenker's fluid for histological study.

Pulmonary Lesions in Infected Mice Which Had Received Normal Horse Serum

In order to determine if the injection of a foreign protein as such would so change the reactivity of mice that pneumococci would localize in the lungs, 201 mice were first injected intraperitoneally with normal horse serum, then alcoholized and exposed to infection by the spray method. Although twenty-five, or 12 per cent of these mice, died of pneumococcus septicemia, no localized lesions in the lungs were present.

Pulmonary Lesions in Passively Immunized Mice

215 mice were passively immunized by a single intraperitoneal injection of Type I antipneumococcus serum, various amounts being employed. They were then alcoholized and sprayed with an homologous culture of Type I pneumococcus. Of the mice so treated, forty-eight, or 22 per cent, died of pneumococcus septicemia, and twelve, or 25 per cent of those dying, showed definite, localized, pulmonary lesions.

In order to determine if pulmonary localization could be produced by the use of immune serum of a heterologous type, mice were similarly injected with Type II antipneumococcus serum, alcoholized and sprayed with a culture of Type I pneumococci. Of the 224 mice so treated, seventeen, or 7 per cent, died of pneumococcus septicemia, but in no instance was there any evidence of pulmonary localization. It appears, therefore, that the injection of heterologous immune serum does not afford sufficient immunity to cause a localization of the pneumococcus infection in the lungs.

Pulmonary Lesions in Actively Immunized Mice

Of 211 mice, previously injected intraperitoneally with varying amounts of a suspension of heat-killed Type I pneumococci and subsequently exposed to a spray of a living culture of pneumococci of the homologous type, thirty-two, or 15 per cent, died of pneumococcus septicemia, and pulmonary localization occurred in four, or 12 per cent of those dying.

Pulmonary Lesions in Mice Actively Immunized by the Injection of Suspensions of Heat-Killed Pneumococci of a Heterologous Type

In order to determine if localization would occur in mice actively immunized by the injection of a suspension of heat-killed pneumococci of a heterologous type, 215 mice were injected with a suspension of heat-killed Type II pneumococci, and 10 days later all the animals were alcoholized and sprayed with a culture of virulent Type I pneumococcus. 52, or 24 per cent of these mice, died of pneumococcus septicemia but in none was there any evidence of an attempt to localize the infection.

DISCUSSION

Table I shows in a condensed form the results obtained in these experiments. It is seen that considerable variation occurred in the frequency of death. At first sight it is difficult to see why only 12 per cent of the animals receiving normal horse serum died, while of those that received immune serum 24 per cent died, and only 7 per cent of those that received heterologous serum. It must be remembered, however, that in infecting animals by the inhalation method it is impossible to control the dosage accurately. There must be great variation in the number of organisms that lodge in different parts of the respiratory tract and also in the number of bacteria that actually invade the tissues. The mice in each experiment were divided into groups, each group of fifteen to twenty mice being treated exactly alike, but the mice of the different groups received different amounts of normal, or immune serum, or heat-killed bacteria. When all these circumstances are taken into consideration it is not surprising that there should have resulted considerable irregularity in the results, so far as protection is concerned, though it is not believed that these results invalidate the conclusions regarding the localization of the infection in the lungs.

TABLE I

The occurrence of pulmonary lesions in mice alcoholized and sprayed with culture of Type I pneumococci after receiving injections of normal serum or homologous or heterologous immune horse serum, or after active immunization by injections of homologous or heterologous heat-killed pneumococcus cultures.

Treated with	No. of mice	No. died	Per cent	No. having pulmonary lesions	Per cent of these dying
Normal horse serum	201	25	12	0	0
Homologous immune horse serum	215	48	24	12	25
Heterologous immune horse serum	224	17	7	0	0
Homologous vaccine	211	32	15	4	12
Heterologous vaccine	215	52	24	0	0

The fact that 25 per cent of the animals which died after receiving homologous immune serum and 12 per cent of those receiving homologous vaccine exhibited localized pulmonary lesions, while none of those previously treated with normal horse serum, heterologous serum or killed bacteria showed pulmonary lesions can hardly be explained by chance alone. The experiments seem to show that slight grades of specific immunity are important in causing the organisms to localize in the lungs and to produce lesions there.

CONCLUSIONS

1. When mice are passively immunized by the intraperitoneal injection of antipneumococcus horse serum or actively by the injection

of heat-killed pneumococcus cultures, and are then alcoholized and sprayed with a culture of pneumococci of the same type as that of the bacteria employed in immunization, a considerable number die with localized lesions in the lungs.

2. If instead of injecting immune serum of the type corresponding to that of the bacteria employed in producing the infection, normal horse serum or immune serum of a heterologous type be injected, or if the animals be previously immunized by the injection of killed pneumococci of a heterologous type, none of the animals which die show any evidence of localization of the infection in the lung.

3. The occurrence of pulmonary lesions in alcoholized mice after spraying with a culture of pneumococci is the consequence of a general immunity of a very mild grade.

[Reprinted from the AMERICAN JOURNAL OF DISEASES OF CHILDREN, July, 1931, Vol. 42, No. 1, pp. 42-51]

INTRAVENOUS VACCINATION WITH HEMOLYTIC STREPTOCOCCI

ITS INFLUENCE ON THE INCIDENCE OF RECURRENCE OF RHEUMATIC FEVER IN CHILDREN*

BY MAY G. WILSON, M.D., AND HOMER F. SWIFT, M.D.

(From the Department of Pediatrics, Cornell University Medical College, the New York Nursery and Child's Hospital and the Hospital of The Rockefeller Institute for Medical Research)

The natural history of rheumatic fever in children is characterized by the repeated recurrence of its many and varied manifestations of activity over a period of years. From the onset until the patient is about 12 years of age there is apparent an increasing susceptibility and after this age a diminishing susceptibility.[1]

If the conception of rheumatic fever is accepted as an allergic or hyperergic phenomenon, and if it is assumed that the many manifestations of activity are due to a state of hypersensitivity of the tissues to some component of streptococci, the period before 12 years may be considered one of predominant hypersensitiveness to streptococci and the period after 12 years as one of gradually increasing hyposensitiveness, possibly due to autodesensitization. It would then seem reasonable to attempt to induce a state of hyposensitiveness during the period of increasing susceptibility. Therefore it was considered worthwhile to attempt to desensitize a group of susceptible children by intravenous vaccination with hemolytic streptococcic vaccine.

The hypothesis on which intravenous vaccination was based, the type and dosage of vaccine used, resulted from investigations by Dr.

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* Presented at the Meeting of the Section of Pediatrics at the New York Academy of Medicine, Dec. 11, 1930.

* This study was made possible by a special grant from the Commonwealth Fund.

[1] Wilson, May G.; Lingg, C., and Croxford, G.: Statistical Studies Bearing on Problems in Classification of Heart Disease, Am. Heart J. 4: 164 (Dec.) 1928.

H. F. Swift and his associates at the hospital of The Rockefeller Institute.[2] These investigations have been published and will not be considered here.

The present investigation covers a four year period of observation from 1927 to 1930, inclusive. A total of 172 children attending the Heart Clinic of the New York Nursery and Child's Hospital were selected for study. Selection was based on the duration of rheumatic disease, the age of the child and the date of last activity. The majority of the children were under 10 years of age; the only children over 10 years of age included, were those with activity in 1927. Approximately one half of the children received intravenous vaccination, and the remaining number, a comparable group, were observed as a control series. Uniform medical supervision and social service follow-up were given to each child of both groups. A detailed and careful record of the symptoms and physical examination were made at least monthly; many of the children were seen weekly. The majority of these children were under observation at the heart clinic from the onset of the disease.

Treatment

The vaccine used was prepared at the hospital of The Rockefeller Institute. It consisted of a heat-killed culture of hemolytic streptococcus strain Q 33 suspended in 0.5 per cent phenolized physiologic solution of sodium chloride so that 1 cc. represented the required dose.[3] The first injection consisted of 250,000 micro-organisms, and each subsequent weekly injection contained double that of the preceding one until the maximum of 10,000,000 was reached, and this dose was repeated until a total of from nine to twelve treatments had been given.

The first series of 20 children was treated in the summer, a second 20 in the fall of 1928, a third series in the winter and a fourth in the spring of 1929. Fifty children of series 1, 2 and 3 received a second course of twelve treatments in the fall of 1929.

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[2] Swift, Homer F.; Hitchcock, C. H.; Derick, C. L., and McEwen, C.: Intravenous Vaccination with Streptococci in Rheumatic Fever, Tr. A. Am. Physicians 1930, vol. 45; Am. J. M. Sc. 181: 1 (Jan.) 1931.

[3] This streptococcus was isolated from the tonsillar exudates of a patient who had suffered for months from severe polyarthritis and carditis with congestive failure.

All of the patients in the first series were hospitalized for forty-eight hours for three successive weekly treatments. The total leukocyte count before and after each treatment did not vary; no febrile or constitutional reaction occurred following vaccination. The succeeding treatments were given in the outpatient department to all of the ambulatory subjects. The mother or visiting nurse recorded the temperature every four hours for forty-eight hours. Patients who required care in bed because of the activity of the disease were given the entire course of treatment in the wards of the New York Nursery and Child's Hospital or at home. In all, 20 patients with activity were treated, which included 15 suffering from chorea and 5 with active carditis.

Reactions were infrequent; 7 children exhibited febrile reactions of from 1 to 2 degrees above normal, with occasional complaints of headache following any single vaccination of variable dosage. Focal reactions were not observed.[4] Little difficulty was encountered in giving these treatments, and no untoward symptoms developed in any child.

RESULTS

The possible influence of the intravenous streptococcal vaccination on the subsequent clinical course exhibited by the treated children may be considered: (1) by comparing the incidence of recurrence before and after treatment and (2) by comparing the incidence of recurrence in the control series during a similar period.

Curves of the seasonal incidence of recurrence were plotted, covering the years from 1927 to 1930 for each series and for the entire groups. The following rheumatic manifestations were recorded as recurrences: active carditis, chorea, polyarthritis, growing pains, pains in the joints, rheumatic eruptions of the skin and rheumatic nodules. The recurrence of one or more manifestations in any child was charted only once, at the onset. These curves represent only the incidence of activity and not the type, number, severity or duration of these recurrences.

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[4] In 1 patient with active carditis a febrile period of 3 degrees above normal for twenty-four hours followed a single vaccination of 500,000 micro-organisms. The evaluation of such reactions occurring coincidentally with treatment is difficult, since such febrile episodes are of frequent occurrence in patients with acute or subacute rheumatic disease.

General inspection of the curves shown in chart 1 indicates that there is an apparent increased incidence in the winter and the spring. There are consistent seasonal peaks for each series during successive years. There is also an increased incidence for the alternate years of 1927 and 1929 in the winter and spring. This may best be seen in the control series during the four year period. Such variability in yearly incidence is characteristic of most infections and must be considered in evaluating therapeutic measures.

A comparison of the trend of the curves of each of the treated and control series shows them to be remarkably comparable for the years 1927 and 1928 before vaccination was instituted. All of these curves show a definite downward trend during the period after treatment; the levels for 1929 and 1930 are lower than those for 1927–1928. The curves of the first and second series, in which treatment was given in the summer and fall, show the lowest levels after treatment. The treatment of children during the winter and spring, which are the seasons of greatest incidence, necessarily includes those showing activity. This perhaps accounts for the slightly higher levels evident in 1930 in the third and fourth series. There is no appreciable difference in the incidence of recurrence noted in 1930 between the third series in which the patients received a second course during 1930, and the fourth series in which the patients received only one course of treatment. Inspection of the curves plotted for both the groups in which treatment was given and the control groups in chart 2 shows again the comparability of the two series before treatment and the lowered incidence after treatment. A comparison of the annual incidence of recurrence among the groups treated and the control groups shows for the former a decreasing yearly incidence of recurrence of 87 per cent in 1927, 78 per cent in 1928, 48 per cent in 1929 and 39 per cent in 1930. This decrease is compared with the almost consistent yearly incidence of 77 per cent, 70 per cent, 79 per cent and 65 per cent in the control group during the same years, respectively. Only about two thirds of the children had completed their course of vaccination by the winter of 1929. It is also of interest to note the low incidence during 1930 for the control group (this year has been characterized as a mild year for most infections). A comparison of the various

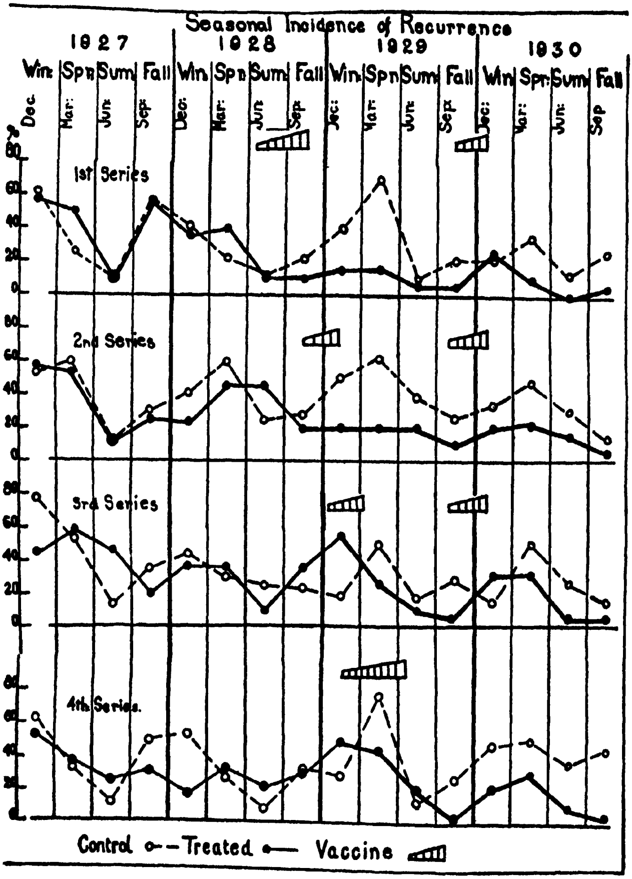

FIG. 1.—Seasonal incidence of recurrence for each series of both groups. A recurrence of one or more manifestations is charted only once at the onset for each child. It is here expressed as the percentage of the total number of recurrences during each season.

manifestations of activity of the disease in the children of both groups for each of the four years (chart 3) shows for 1927 and 1928 a comparable distribution of the various types of activity, while for the years 1929 and 1930 there is a definite decrease in the incidence of carditis, chorea and arthritis.

During the period of from sixteen months to two years following the completion of the course of vaccination, 44 children suffered some recurrence as compared with 69 children of the control group for the same period.

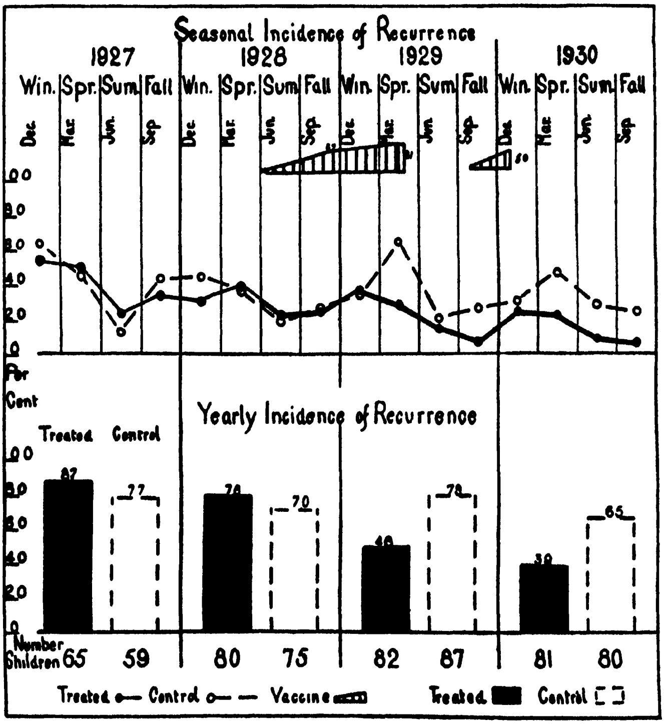

FIG. 2.—*A* represents seasonal incidence of recurrence for the total vaccinated and control groups; *B*, the yearly incidence of recurrence for both groups.

In chart 4, it may be seen that the children of the control group exhibited many more manifestations of activity of the disease. Possibly this indicates a greater severity of infection. The possible influence of treatment on the severity or duration of these recurrences is difficult to estimate, since the natural course of the disease is characterized by variation in duration and severity of all of the manifestations. It is also difficult to evaluate the influence of vaccination during activity, since the clinical course of any of the manifestations is self-limited. It is of interest to note that no untoward reactions developed following vaccination.

From these observations, one may conclude that the group in which treatment was given and the control group were comparable both as to type of activity and incidence of recurrence in 1927 and 1928 before treatment. The vaccinated series exhibited a diminished total incidence of recurrence in 1929 and 1930 as compared with the two previous years and also with the control series for the same years. Forty-five per cent of the treated patients remained free from recurrence for a period of from sixteen months to two years following treatment, as compared with only 18 per cent among the control group for the same period.

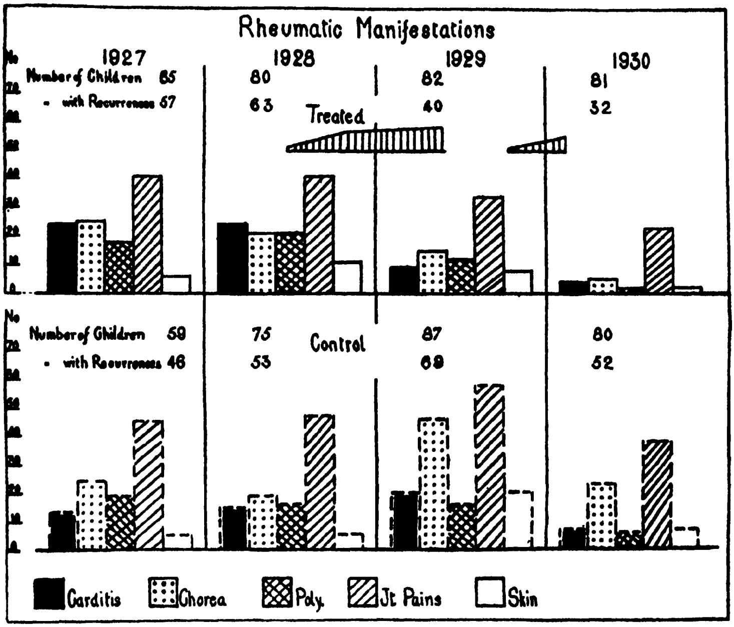

FIG. 3.—Manifestations of recurrence in children of both groups for the years 1927 to 1930.

A comparative analysis of the ages of the children of the two groups in 1929–1930 shows that in 70 per cent of the treated children and in 78 per cent of the control series the subjects were 12 years of age or less. Fifty per cent of the patients who were treated and 80 per cent of the controls in this age period suffered some recurrence. This reduction in the incidence of recurrence during the age period of greatest susceptibility in the treated series is noteworthy. In half of the children of both groups the duration of the disease was from one to three years.

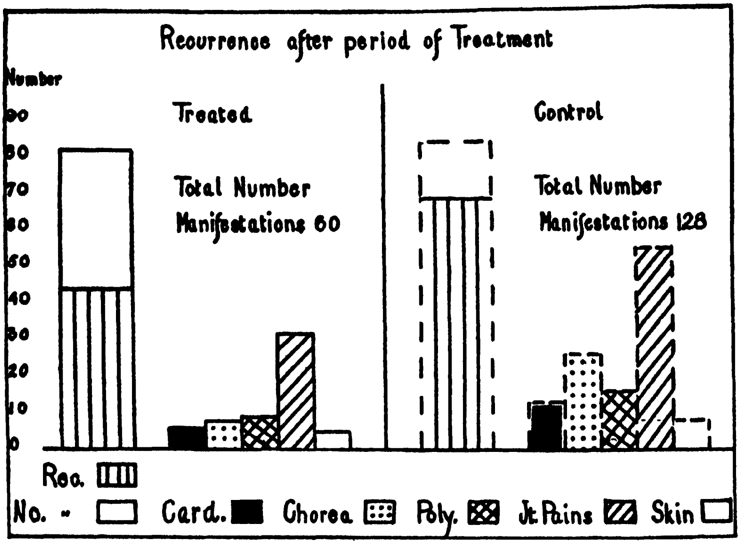

FIG. 4.—Various manifestations of recurrence for the period of from sixteen months to two years following vaccination. Recurrence after the period of treatment.

A consideration of the presence of other possible factors that may be responsible for the difference in the incidence of recurrence in the two groups revealed the following: In the treated series, 75 per cent of the patients had been subjected to tonsillectomy. In the control series, 71 per cent had had tonsillectomy before 1929. The incidence of tonsillectomy was approximately the same in the children having recurrences and in those free from recurrences in both of these groups.

Clinical and roentgenographic examination limited to 54 of these children did not disclose evidence of diseased sinuses.

A consideration of the possible relation of infections of the upper part of the respiratory tract to the incidence of recurrence of rheumatic activity on analysis of the case histories revealed that a similar incidence of infections of the upper part of the upper respiratory tract was observed in both the control and the treated groups during the period of observation. There did not seem to be any close relationship between the occurrence of infections of the upper part of the respiratory tract and the onset of rheumatic activity in either of the groups.

Examination of the serum[5] of 56 treated children for the presence of agglutinins and precipitins gave uniformly negative results.

Comparative cutaneous tests with various strains of streptococcic filtrates given before treatment and for various periods after treatment gave inconclusive results. The incidence of positive reactions to certain strains of streptococci and nucleoproteins in the treated and in the control groups was comparable.[6] There did not seem to be any change in the reactivity of the treated children as judged by these cutaneous tests.

COMMENT

In the discussion of the causal relationship of intravenous vaccination to the diminished incidence of recurrence observed in the vaccinated children, the following possibilities will be considered: 1. The diminished incidence of recurrence bears no relation to the vaccination and is merely coincidental. 2. Vaccination caused the stimulation of specific antibodies with resulting increased immunity, which has been either the result of nonspecific protein irritation or of a specific immunization with the hemolytic streptococci. 3. The intravenous vaccination resulted in a desensitization of the patient to streptococci, and was not dependent on specific immunization.

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[5] Examinations were made by Dr. C. H. Hitchcock at the hospital of The Rockefeller Institute.

[6] Tests of the skin with hemolytic streptococcus Q 33 in a solution of 1:100 gave negative results in 35 per cent of the cases in which treatment was given and in 30 per cent of the controls; with nucleoprotein streptococcus P 43, 0.1 cc.; tests with 0.001 mg. were negative in 35 per cent of the patients who were treated and in 42 per cent of the controls; tests with green streptococcus O 28B, in a solution of 1:10 gave negative results in 53 per cent of the cases in which treatment was given and in 40 per cent of the controls.

One cannot dismiss the fact that many rheumatic children have from three to five years of temporary freedom from recurrence, and consequently the diminished incidence of recurrence observed may have been the result of a natural acquired immunity unrelated to therapy. However, the statistical evidence, though based on relatively few numbers, as well as the observations in the control series, favors the view that the intravenous vaccination may bear some relationship to the diminished incidence of recurrence observed. The period of observation of only from sixteen months to two years after vaccination does not assure the permanency of such influence. The data presented does not indicate whether yearly courses of vaccination are desirable.

Assuming that intravenous vaccination was the factor influencing the clinical course of the disease, the evidence here presented does not reveal the underlying mechanism. Stimulation of the formation of specific antibodies or nonspecific stimulation of resistance following vaccination must be considered. Although the usual febrile evidence of nonspecific protein reaction was not observed, it is impossible definitely to rule out such nonspecific reaction not clinically apparent because of the small dosage. Control studies with other antigens, such as typhoid vaccines, might be found to exert a similar influence. An investigation of the effect of intravenous vaccination with typhoid vaccines in similar doses is under way at the New York Nursery and Child's Hospital and will be the subject of a subsequent communication. Up to the present we have not observed febrile nonspecific reactions to protein following similar doses of typhoid vaccines. Increasing the dosage to 15,000,000 and 20,000,000 micro-organisms gave definite characteristic nonprotein reactions.

If the results reported were due to a specific effect, one might possibly have expected febrile reactions with increasing doses; hence the absence of such reactions in this group of patients is noteworthy. We are also treating a group of children with autogenous vaccine administered in similar doses. Up to the present time we have not observed reactions following intravenous vaccination. This absence may have been due either to the small doses employed or to a relatively nonreactive state of the patients in the stage during which they were treated. The absence of agglutinins and precipitins from the blood serum is of negative importance, for active immunity has been observed in the absence of demonstrable circulating specific antibodies.

The assumption that the lowered incidence of recurrence observed in the vaccinated group was due to a diminished susceptibility resulting from a change in reactivity of the tissues may be explained by the conception of the disease as an allergic (hyperergic) phenomenon. Cutaneous tests with certain streptococcal filtrates, however, did not demonstrate a diminished reactivity of the skin comparable to that observed by Swift and his co-workers in studies on experimental hyperergy in rabbits.[7] Because of the difficulty at present of accurately evaluating all of the possible factors involved, it would seem that the evidence here presented does not warrant definite conclusions concerning this hypothesis.

Final judgment as to the causal relation of intravenous vaccination with hemolytic streptococcal vaccine to the diminished incidence of recurrence observed must await the results of further investigation. It would seem best at the present time to restrict the therapeutic application of intravenous vaccination in rheumatic children to controlled investigation.

SUMMARY

The seasonal incidence of recurrence of rheumatic activity among 172 children of the susceptible age group observed for a period of four years is presented.

About one half of these children received intravenous vaccination with hemolytic streptococcal vaccine. The remaining numbers were observed as a control group.

The incidence of recurrence and manifestations of activity in both groups were comparable during the two years 1927 and 1928 before vaccination was given.

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[7] Swift, Homer F., and Derick, C. L.: Reactions of Rabbits to Non-Hemolytic Streptococci: II. Skin Reactions in Intravenously Immunized Animals, J. Exper. Med. 49: 883 (May) 1929, Derick, Clifford L., and Swift, Homer F.: Hyperergic Tissue Response to Non-Hemolytic Streptococci, Proc. Soc. Exper. Biol. & Med. 25: 222, 1927. In the dosage of intravenous injections of vaccine and in the substances used for intracutaneous injection, the two sets of observations are not strictly parallel.

The yearly incidence of recurrence in the treated group was less than in the control group during the two years 1929 and 1930 after treatment.

Forty-five per cent of the treated children as compared with 18 per cent of the controls were free from recurrence for periods of from sixteen months to two years after treatment.

The causal relation of intravenous vaccination with hemolytic streptococcal vaccine to the diminished incidence of recurrence observed is discussed.

I wish to acknowledge my indebtedness to my associates in the Heart Clinic and to the House Staff of the New York Nursery and Child's Hospital for their assistance. I also wish to express my appreciation of the intelligent cooperation given by the social service department of the hospital.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, October 1, 1931,
Vol. 54, No. 4, pp. 453–461]

CULTIVATION OF VACCINE VIRUS FOR JENNERIAN PROPHYLAXIS IN MAN

BY T. M. RIVERS, M.D.

WITH THE TECHNICAL ASSISTANCE OF S. M. WARD

(From the Hospital of The Rockefeller Institute for Medical Research)

PLATES 45 TO 47

(Received for publication, June 15, 1931)

In a previous communication (1) a simple medium for the cultivation of vaccine virus was described. The results of the work reported at that time clearly indicate that vaccine virus is capable of multiplication in the presence of minced chick embryo tissue suspended in Tyrode's solution. The experiments, however, were conducted with a neurovaccine virus, and, although the active agent engendered in cultures caused typical vaccinal lesions in rabbits, it was deemed best not to test it in human beings. To obtain a culture virus for Jennerian prophylaxis in man, it seemed advisable to adapt a dermal strain of vaccine virus to our method of cultivation. The present paper deals with the results of this work.

Methods and Materials

Virus.—Vaccine virus, Lot 611, prepared by the New York City Board of Health was used to initiate the cultures. In a letter to me, the original source of the virus is described by Mr. Chas. R. Tyler.

"Regarding the history of our strain of vaccine virus: the following statement appears in the Yearly Report of the New York City Board of Health for 1874–75.

'We began vaccinating with virus of the same stock as that which had been supplied by the late Dr. Loines of the Eastern Dispensary and myself for about 5 years and which had been used and sold by him for about 20 years previously. This virus was originally obtained from England by Dr. Loines and in all probability was descended from the stock furnished by Jenner. As it always developed characteristic Jennerian vesicles and as it always thoroughly protected from smallpox those upon whom it was used, Dr. Loines never thought favorably of employing any other.'

"The same strain of virus has been in use since the time of this report. The virus has been 'humanized' at various times, usually from one to three times yearly."

Tissues.—Embryonic tissue was obtained from eggs, incubated 9 to 12 days, that had been opened according to the method of Carrel and Rivers (2) or that of Eagles and McClean (3). If the embryos were contaminated by yolk, they were thoroughly washed in sterile Tyrode's solution before being minced. Furthermore, the eyes were removed, because the pigment granules contained in them often led to confusion when smears were made to determine the sterility of the cultures. Finally, the embryos were placed in a sterile watchglass contained in a Petri dish, finely minced with scissors, and then distributed in proper amounts into flasks by means of a pipette.

Tyrode's Solution.—Tyrode's solution prepared according to the following formula and sterilized by filtration was used: NaCl, 8 gm.; KCl, 0.2 gm.; $CaCl_2$, 0.2 gm.; $MgCl_2$, 0.1 gm.; NaH_2PO_4, 0.05 gm.; $NaHCO_3$, 1.0 gm.; glucose, 1.0 gm.; water *q.s.* 1000 cc. During the preparation and filtration of the solution, CO_2 is liberated, and the pH is found to be between 8.0 and 8.4. When living tissue is added to the solution, however, CO_2 is supplied by it and the pH becomes less alkaline reaching a point compatible with the survival of the embryonic tissue and the multiplication of the virus.

Containers.—Containers for the production of vaccine virus in large amounts must be of sufficient size and easy to handle. Furthermore, they must permit aeration and prevent contaminations and evaporation. Flasks (collar flasks) designed to meet these requirements were described in a previous paper (1) and have been found to be satisfactory for this work. Two sizes have been used, one with approximately the dimensions of a 50 cc. Erlenmeyer flask, the other with approximately the volume of a 250 cc. Erlenmeyer flask. In the small container 5 cc. of medium were placed, while in the large one 15 cc. were used. Such small amounts were placed in each flask in order to have the medium distributed as a thin layer. In case collar flasks cannot be obtained, 50 cc. and 250 cc. Erlenmeyer flasks make good substitutes. All glassware used in this type of work should be made of an alkali-free resistant glass, *e.g.*, pyrex.

Preparation of Cultures.—Approximately 1 gm. of minced chick embryo tissue to each 5 cc. of Tyrode's solution was used. Proper amounts of tissue suspended in its vehicle were distributed in flasks. The medium was then inoculated with 0.25 cc. of virus emulsion, the mouth of each flask containing its cotton plug was securely covered with several layers of tin-foil, and the cultures were incubated at 37°C. for 5 days. New cultures were made by direct transfer of 0.25 cc. of an old culture into flasks of fresh medium. In this simple manner, vaccine virus can be propagated through an indefinite number of culture generations. One should be careful not to use too much tissue for a given amount of Tyrode's solution, inasmuch as it appears that an optimum ratio between tissue and fluid exists for the maximal multiplication of virus.

Tests for Sterility of the Cultures.—Early in the work, the medium was tested for sterility before it was inoculated with virus. During the interval (48 hours), the prepared flasks were stored at +5°C. Later these preliminary tests were omitted to advantage. Two separate batches of media were prepared for each transfer in order to escape the possibility of having the work delayed by bacterial contaminations. After the cultures had been incubated for 3 days, a small amount of material was removed from each one and seeded in broth tubes and on blood agar plates. After the virus cultures had been incubated for 5 days, smears from each one were prepared, stained, and examined for bacteria. The material in the flasks that was found by cultural tests and smears to be free from bacteria was used for transfers and animal inoculations. Numerous flasks have been handled in the manner described above and only 1 in 12 became contaminated with bacteria.

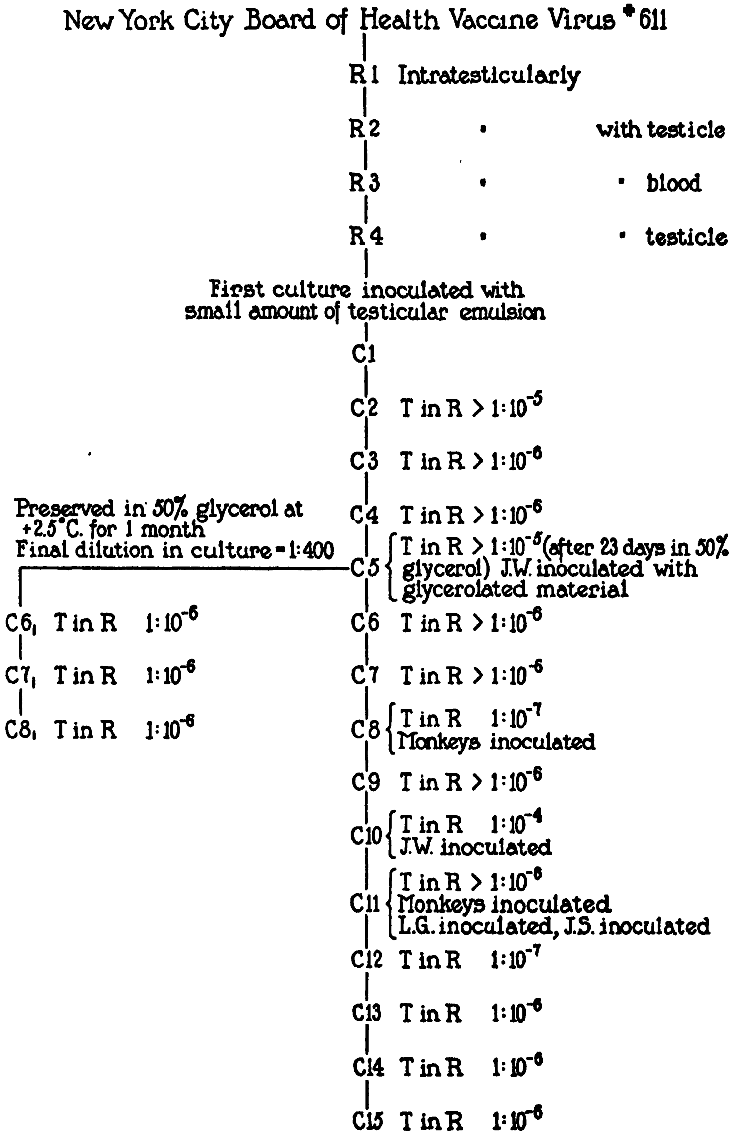
Tests for Potency of Virus.—Cultures for titration were ground in sterile mortars, and then appropriate dilutions were made with Locke's solution. A fresh pipette was always used for each successive dilution. 0.25 cc. of each dilution were injected into the shaved skin or were smeared on the scarified shaved skin of rabbits. In addition to rabbits, monkeys and human volunteers were employed for the potency tests of the virus.

Preservation of the Virus.—Many workers have stated that culture virus is difficult to preserve. This is true of viruses cultivated in certain kinds of media. It must be borne in mind, however, that no vaccine virus keeps indefinitely above 0°C. Furthermore, when glycerol is used, care must be exercised in selecting one that will not inactivate the virus. Merck's (P-W-R Analytical Chemicals) glycerol has been found suitable for this work.

EXPERIMENTAL

The vaccine virus distributed by the New York City Board of Health is prepared on calves, ripened in glycerol at about −8°C., and then treated with brilliant green. The bacterial count in such material is low. Indeed, at times, the virus seems to be entirely free from viable microorganisms. A specimen from Lot 611 was obtained from the Board of Health Laboratories, and with it cultures were initiated. At first it appeared that we had been fortunate enough to obtain a virus free from bacteria, but soon it was discovered that a very slow-growing diptheroid was present as a contaminant. Consequently we were faced with the problem of how to rid the virus cultures of the bacteria. Filtration was first considered as a means of accomplishing this, but it was not attempted because vaccine virus filters with difficulty. Then we decided to try passage through rabbits in the following manner.

Rabbit 1 received in each testicle 1 cc. of a culture containing diphtheroids and vaccine virus. 4 days later the testicles were removed and emulsified with Locke's solution. 1 cc. of the emulsion was injected into each testicle of Rabbit 2. After


New York City Board of Health Vaccine Virus #611
R1 Intratesticularly
R2 " with testicle
R3 " " blood
R4 " " testicle
First culture inoculated with small amount of testicular emulsion
C1
C2 T in R > 1:10^-5
C3 T in R > 1:10^-6
C4 T in R > 1:10^-6
C5 T in R > 1:10^-5 (after 23 days in 50% glycerol) J.W. inoculated with glycerolated material
Preserved in 50% glycerol at +2.5°C. for 1 month
Final dilution in culture = 1:400
C6₁ T in R 1:10^-6
C7₁ T in R 1:10^-6
C8₁ T in R 1:10^-6
C6 T in R > 1:10^-6
C7 T in R > 1:10^-6
C8 T in R 1:10^-7 Monkeys inoculated
C9 T in R > 1:10^-6
C10 T in R 1:10^-4 J.W. inoculated
C11 T in R > 1:10^-6 Monkeys inoculated L.G. inoculated, J.S. inoculated
C12 T in R 1:10^-7
C13 T in R 1:10^-6
C14 T in R 1:10^-6
C15 T in R 1:10^-6

TEXT-FIG. 1. Summary of experiments in which a dermal strain of vaccine virus was adapted to a culture medium consisting of minced chick embryo suspended in Tyrode's solution. T in R = titer in rabbit.

a lapse of 4 days, blood (4) was removed[1] aseptically from the heart of Rabbit 2 and immediately injected in 1 cc. amounts into the testicles of Rabbit 3. This animal developed fever on the 4th day, and small hard nodules were felt in the testicles. At this time the testicles were removed and emulsified with Locke's solution. 1 cc. of the emulsion was inoculated in each testicle of Rabbit 4. After 4 days these testicles were removed and ground in a mortar without sand. Approximately 20 cc. of Tyrode's solution were thoroughly mixed with the macerated tissue. The emulsion was tested for the presence of bacteria and was found to be sterile. Then small amounts (0.25 cc.) of the supernatant material from a centrifuged specimen of the emulsion were used to initiate a series of virus cultures (see Text-fig. 1).

Having rid the dermal vaccine virus of bacteria it remained for us to determine whether it was possible to adapt such a strain to our method of cultivation. Proceeding in the manner described above, we have been able to carry the virus through an uninterrupted series of 15 culture generations (Text-fig. 1), and there is no reason to suppose that it cannot be cultivated indefinitely. The records of the titrations of the virus shown in Text-fig. 1 leave no room for doubt concerning the multiplication of the active agent. Furthermore, fresh cultures have been initiated with glycerolated material (Text-fig. 1) stored for 30 days at +2.5°C. Such facts make it evident that vaccine virus is capable of indefinite propagation in cultures without the intervention of animal passage.

Reactions in Animals

It has been shown that a dermal strain of vaccine virus is capable of multiplication in a medium consisting of minced chick embryo suspended in Tyrode's solution. The question as to whether the virus has been altered by such treatment naturally arises. To investigate this matter, rabbits and monkeys were employed.

Material from each set of cultures, with the exception of the 1st, has been tested by intradermal inoculations in the shaved skin of rabbits. The reactions caused by the virus have not been altered through the process of cultivation, and have always been similar to those produced by a potent strain of vaccine virus unassociated with bacteria. This fact is illustrated by Fig. 1 that portrays lesions following intradermal injections of different dilutions of a culture in the 14th generation. In addition to intradermal injections, inoculations on the scarified shaved skin of rabbits have been made from time to time. Following such inoculations, typical vaccinal lesions unassociated with hemorrhage and necrosis developed. Fig. 2 represents lesions produced by different dilutions of a culture in the 6th generation. Finally, it has been shown that the culture virus smeared on the scarified skin of monkeys (*Macacus rhesus*) induces typical vaccinal lesions that heal rapidly without resultant injury to the animals.

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[1] All operations were performed under ether anesthesia.

From the above experiments it is obvious that the culture virus caused typical vaccinal lesions in animals. We then became interested in determining whether the active agent had undergone immunological changes. Consequently the following cross immunity experiments were performed.

Rabbits inoculated respectively with virus from the 6th, 8th, 10th, and 11th culture generations were allowed to recover. Then, they and 2 normal stock rabbits were inoculated with New York City Board of Health vaccine virus, Lot 617. The recovered animals were refractory while the normal ones developed typical vaccinal lesions.

The above experiment indicates that repeated cultivation of the virus in a medium consisting of minced chick embryo suspended in Tyrode's solution did not alter its antigenic properties.

Reactions in Human Volunteers

Not infrequently we receive requests for vaccination against smallpox. Since there was no valid reason for not using the culture virus in human beings, we decided, with the consent of the patients or of their parents, to substitute in certain instances the culture vaccine for calf lymph. The results obtained in 3 children are detailed below.

J. W., aged 2 years, *May 2*, was vaccinated at 2 points on the outer surface of the upper left arm. The superior inoculation was made with material from a 5th-generation culture that had been preserved in 50 per cent glycerol at +2.5°C. for 28 days. The inferior inoculation was made with material from a 10th-generation culture that had been preserved in 50 per cent glycerol at +2.5°C. for 2 days. The method of vaccination consisted of a small amount of the virus being smeared over an area of skin previously superficially scarified. *May 3*, superficial marks at points of inoculation. *May 4*, no change. *May 5*, 9:00 a.m., both lesions, the lower slightly more than the upper, show some swelling and elevation. 2:30 p.m., there have appeared, particularly over the area of the lower inoculation, crops of small vesicles (Fig. 3). No fever. *May 6*, lesions well developed. Both are small, the upper being about 0.5 cm. in diameter, the lower about 1.0 cm. (Fig.

4). Temperature rose to 101.2°F. *May 7*, lesions have increased in diameter (Fig. 5). Temperature rose to 101.6°F. *May 8*, lesions are still increasing in size and are now pustular (Fig. 6). Temperature rose to 102°F. Further development of the lesions not observed, because the child had to leave town with its parents.

L. G., aged 22 months, *May 11*, was vaccinated at one point on the outer surface of upper left arm with material from an 11th-generation culture that had been preserved in 50 per cent glycerol at +2.5°C. for 11 days. *May 12*, no evidence of inflammation. *May 13*, vaccinated area red but not elevated (Fig. 11). *May 14*, at point of inoculation, there is a red papule (Fig. 12). *May 15*, several small vesicles have appeared (Fig. 13). *May 16*, vesicles have coalesced and are beginning to be pustular. Narrow primary areola is present (Fig. 14). *May 17*, lesion somewhat larger, no induration, no fever. *May 18*, lesion has increased in size and is beginning to dry in center (Fig. 15); no fever. *May 19*, pustule larger (Fig. 16), no induration, no fever. *May 20*, pustule larger, drying; secondary areola 3 mm. in width; slight amount of induration (Fig. 17). Temperature rose to 100.4°F. *May 21*, pustule drying, secondary areola 4 mm. in width, induration more marked (Fig. 18). Temperature 100.0°F. *May 22*, secondary areola 1 cm. in diameter, induration more marked (Fig. 19); no fever. Patient was reinoculated on right arm with New York City Board of Health vaccine virus, Lot 617. *May 23*, primary vaccination: secondary areola has almost disappeared, induration less (Fig. 20). Secondary vaccination: skin at point of inoculation red. No fever. *May 25*, primary vaccination: lesion covered by dry scab; secondary areola and induration no longer present (Fig. 21). Secondary vaccination: slightly red. *May 26*, primary vaccination: only a dry scab remains. Secondary vaccination: negative. *May 28*, primary vaccination: the scab will drop off in a few days (Fig. 22). Secondary vaccination: negative. Patient in excellent condition and discharged from the hospital. The virus with which this patient was reinoculated was shown to be potent by a successful vaccination of a normal person. Thus, the lack of response to the secondary inoculation indicates that a state of immunity had been induced by the primary vaccination.

After it had been demonstrated that the culture virus produces without danger a typical vaccinia in man and that it induces an immunity against calf lymph, we decided, in view of the fact that the culture vaccine seemed to possess a greater potency than does ordinary calf lymph, to compare the action of the two kinds of active agent.

M. S., aged 11 months, and J. S., aged 22 months, were vaccinated; the former with New York City Board of Health virus, Lot 617, that had been stored in 50 per cent glycerol below 0°C. for more than a year, the latter with virus from an 11th-generation culture that had been preserved in 50 per cent glycerol at +2.5°C. for 21 days. Typical vaccinia developed in both children who showed no evidences of illness except a local reaction and a slight elevation of temperature, 101.5°F. Certain differences, however, were noted in the course of the infection in the 2 patients, *viz.*, the child who was inoculated with the culture virus had a more prolonged febrile reaction and developed more redness and induration associated with a few secondary vesicles around the vaccinal pustule than did the infant who received the calf lymph (Figs. 7 to 10).

The experiment just described and others that will not be presented at this time indicate that the culture virus is very potent. The fact that fresh culture vaccine produces considerable reaction is not surprising, inasmuch as it has long been known that a "green" virus is more likely to give rise to redness, induration, and secondary vesicles than is one properly aged. Therefore, the culture virus should be allowed to ripen before it is used for vaccination of human beings.

DISCUSSION

The medium employed for the *in vitro* cultivation of vaccine virus is exceedingly simple, but one must remember that it contains living cells. In spite of recent reports (5) to the contrary, no one has definitely shown (6, 7) that vaccine virus is capable of pullulation in the absence of surviving susceptible cells.

Several kinds of media have been devised for the production of vaccine virus. The one described in this paper, however, is the best of the lot already proposed, because, inasmuch as neither serum nor plasma is used and since the tissue consists of minced chick embryo, it is the least likely to become contaminated by an extraneous virus injurious to man. Furthermore, the virus produced in this manner remains potent in 50 per cent glycerol at +2.5°C. for at least a month. More information, however, concerning the length of time the culture vaccine remains active under a variety of conditions should be obtained and to this end experiments are now being conducted.

SUMMARY

1. A dermal strain of vaccine virus has been adapted to a simple culture medium consisting of minced chick embryo suspended in Tyrode's solution.

2. The bacteria-free culture virus, thus obtained, produces in lower animals and in man typical vaccinia that renders them refractory to infection with ordinary vaccine virus harvested from calves.

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EXPLANATION OF PLATES

PLATE 45

FIG. 1. Lesions produced in a rabbit by intradermal inoculations (0.25 cc.) of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilutions, respectively, of a 14th-generation culture of vaccine virus. × 1.

FIG. 2. Lesions induced in a rabbit by different dilutions (undiluted, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) of a 6th-generation culture of vaccine virus. × 1.

PLATE 46

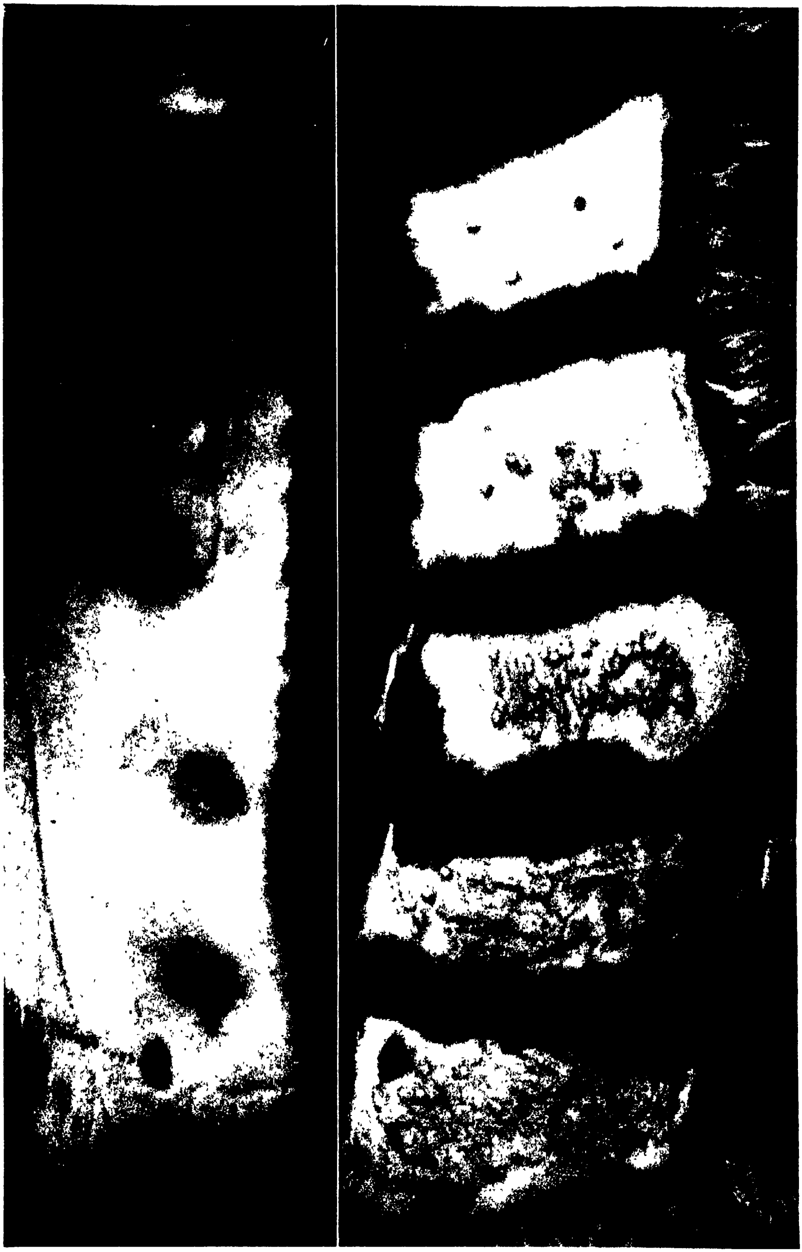
FIGS. 3 to 6. 3rd-, 4th-, 5th-, and 6th-day vaccinal lesions, respectively, produced in J. W. by culture virus: the upper resulted from an inoculation of a 5th-generation culture, the lower from an infection with a 10th-generation culture. × 1.

FIGS. 7, 9. 6th- and 9th-day vaccinal lesions, respectively, produced in M. S. by New York City Board of Health virus, Lot 617. Compare with Figs. 8 and 10. × 1.

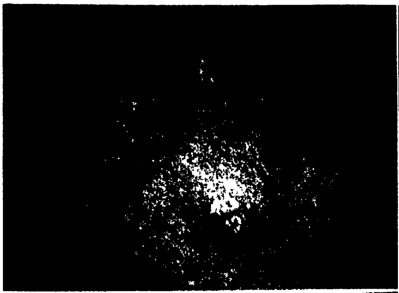
FIGS. 8, 10. 6th- and 9th-day vaccinal lesions, respectively, induced in J. S. by an 11th-generation culture. Comparison of these reactions with those in Figs. 7 and 9 shows that the culture vaccine, only 21 days old, caused more redness and secondary vesicles than did the City Board of Health virus that had been aged for more than a year. × 1.

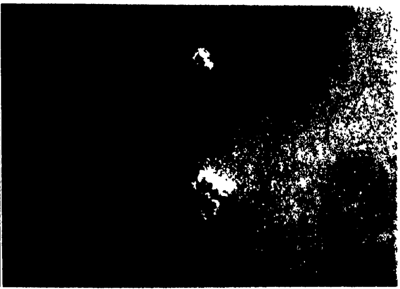
PLATE 47

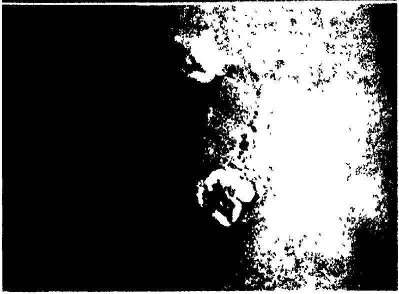
FIGS. 11 to 22. 2nd-, 3rd-, 4th-, 5th-, 7th-, 8th-, 9th-, 10th-, 11th-, 12th-, 13th-, and 18th-day vaccinal lesions, respectively, produced in L. G. by a 10th-generation culture. The photographs portray the complete evolution of a vaccinal lesion induced in man by a dermal strain of virus adapted to *in vitro* cultivation. × 1.

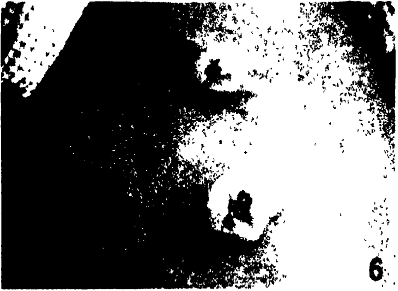


(Rivers: Vaccine virus for prophylaxis)









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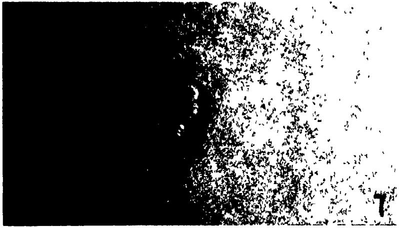

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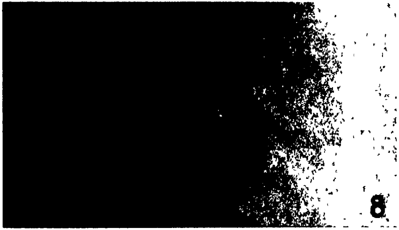

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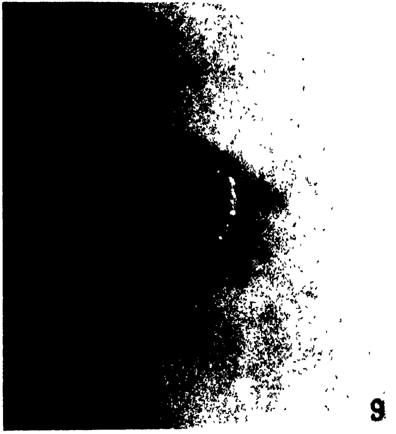

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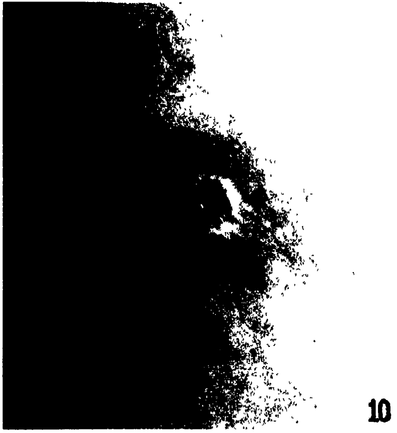

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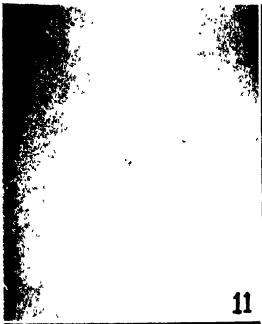
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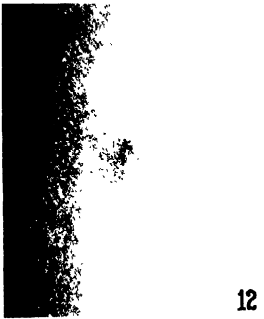
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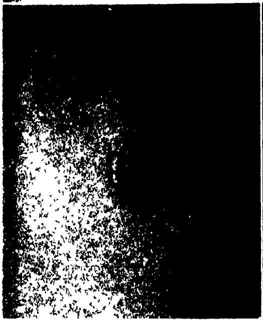

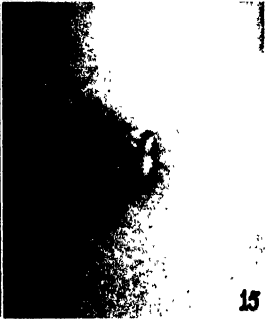
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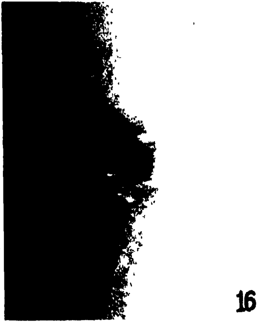
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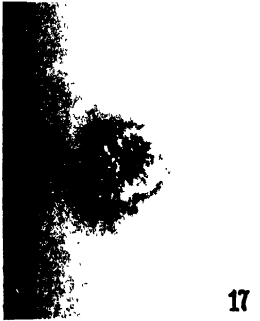
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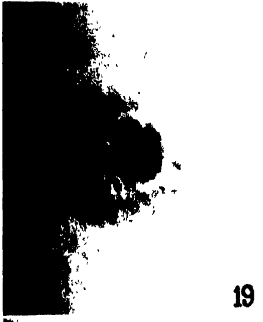
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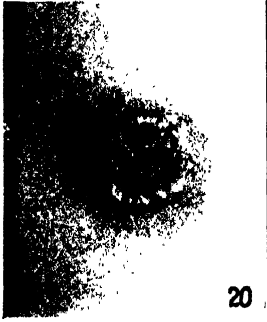
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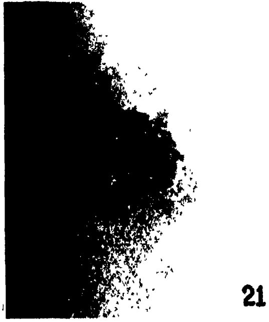
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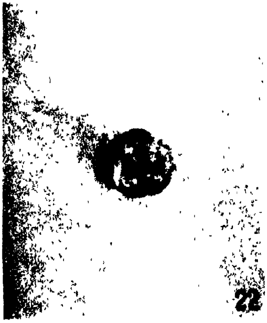
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DIE HORTEGASCHEN ZELLEN IM „ENTZÜNDLICHEN REAKTIONSKOMPLEX" DER BORNASCHEN KRANKHEIT

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(Eingegangen am 15. Januar 1931)

Neuere Untersuchungen von *Nicolau* und *Galloway*[1], *Zwick*[2], *Dobberstein*[2], *Seifried* und *Spatz*[4] zeigen, daß die Wucherungen der Neuroglia im entzündlichen Reaktionskomplex der Bornaschen Krankheit neben den vasculären und perivasculären Infiltraten einen hervorstechenden Befund darstellen (s. Abb. 1). Nach dem häufigen Vorkommen sog. Stäbchenzellen, die im Bereiche der knötchenförmigen und diffusen Gliaproliferationen schon bei Anwendung gewöhnlicher Färbemethoden, besonders der Nissl-Färbung hervortreten (s. Abb. 1), ist zu erwarten, daß auch die *Hortega*schen Zellen eine wesentliche Rolle spielen. Darauf und auf die Notwendigkeit, durch Anwendung entsprechender Spezialmethoden nähere Aufschlüsse über die Beteiligung der beiden Hauptgliazelltypen (Astrocyten und *Hortega*sche Zellen) bei dieser Krankheit zu gewinnen, ist bereits von *Seifried* und *Spatz*[4] hingewiesen worden. Diese Lücke in der Kenntnis der feineren Histologie der Bornaschen Krankheit auszufüllen, ist Zweck der vorliegenden Arbeit. Kurz vor Abschluß meiner Untersuchungen ist mir die Veröffentlichung von *Bratianu* und *Llombard*[5] bekannt geworden, die in der Zwischenzeit derselben Fragestellung nachgegangen sind.

SCHRIFTTUM

Die Kenntnis der *Hortega*schen Zellen, der sog. „Mikro- oder Mesoglia" im Z.N.S.* des Menschen und der Säugetiere verdanken wir den Arbeiten *P. del Rio Hortegas*[6]. Aus der Gruppe der sog. „adentrischen Zellen" gelang es ihm, mit seiner Versilberungsmethode (Silberreduktion an der Oberfläche von Strukturen) die Mikrogliazellen beinahe elektiv darzustellen. Sie zeigen bei einer auffallenden

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* Z.N.S. = Zentralnervensystem.

Schmalheit des perinucleären Protoplasmas und beim Vorhandensein spärlicher Hauptfortsätze eine reiche und feine Verästelung. Es lassen sich rein bipolare und mehr multipolare Typen unterscheiden. Durch diese besondere morphologische Eigentümlichkeit, die ovale, längliche oder winkelig abgeknickte Gestalt ihres Kernes weichen sie wesentlich von den neurogliösen Elementen, insbesondere den Astrocyten ab. Die von *Hortega*[6] vertretene Ansicht der mesodermalen Abstammung seiner Zellen, die er später (im Gegensatz zu den Nervenzellen = 1. Element und den ektodermalen Stützgewebszellen, den wahren Gliazellen = 2. Element) als sog. 3. Element bezeichnet, wird von *Metz* und *Spatz*[7] bestritten, während *Gallego*[8] sowie *Bratianu* und *Llombard*[5] sich ihr anschließen. Solange diese Frage ungeklärt ist, ist die Bezeichnung „Mesoglia" jedenfalls nicht gerechtfertigt (*Metz* und *Spatz*). Der Name „Mikroglia" andererseits ist unzutreffend, weil diese Zellen—wenn pathologisch verändert—geradezu ungewöhnliche Ausmaße annehmen können (*Hortega*, *Metz* und *Spatz*, *Gallego* u. a.). Es empfiehlt sich deshalb, nach dem Vorschlage von *Metz* und *Spatz*[7], sie nach ihrem Entdecker als *Hortega*sche Zellen zu bezeichnen.

Das Verhalten der *Hortega*schen Zellen bei verschiedenen Krankheiten des Z.N.S. ist bereits wiederholt Gegenstand von Untersuchungen gewesen, so bei der Tollwut, Poliomyelitis infectiosa, Hundestaupe, Paralyse u. a. Nach den bei diesen Krankheiten erhobenen Befunden unterliegt es keinem Zweifel, daß den *Hortega*schen Zellen bei entzündlichen Prozessen die Eigenschaft der Proliferation und Hypertrophie zukommt. Die *Nissl*schen Stäbchenzellen bei der Paralyse, das *Spielmeyer*sche Strauchwerk in der Kleinhirnrinde, bestimmte Zellformen bei der tuberkulösen Meningitis und bei den genannten Viruskrankheiten des Z.N.S. sind nichts anderes als hypertrophierte *Hortega*sche Zellen. Sie besitzen außerdem die Fähigkeit, unter normalen und pathologischen Verhältnissen lipoide Stoffe, Eisenpigment u. a. aufzunehmen und sich zu Gitterzellen umzubilden.

Bei der Bornaschen Krankheit fehlen noch nähere Einblicke in das Verhalten, das zeitliche Auftreten der Proliferation, die Funktion und die Beteiligung der *Hortega*schen Zellen an der Bildung der knötchenförmigen und diffusen Gliawucherungen. Von *Bratianu* und *Llombard* ist lediglich in älteren Fällen von Bornascher Krankheit ihre Anwesenheit und ihre Modifikation festgestellt worden.

Material und Methodik

In Ermangelung frischen Gehirnmaterials von Bornascher Krankheit beim Pferde wurden für die vorliegenden Untersuchungen experimentell erzeugte Fälle dieser Krankheit beim Kaninchen verwendet. Die Verwendung dieser Tierart schien auch deshalb zweckmäßig, weil bei ihr die *Hortega*schen Zellen leicht dargestellt werden können, während dies beim Pferde nicht zutrifft. Im ganzen wurde das Z.N.S. von 12 Kaninchen untersucht, die intracerebral mit Bornavirus* infiziert und 3, 5, 10, 15, 20, 25, 28, 30, 32, 34, 35 und 38 Tage nach der Impfung mit Chloroform oder durch intravenöse Injektion von Luft getötet worden waren. Der Virusstamm besaß normale Virulenz: Die Tiere erkrankten nach der üblichen Inkubationsfrist von durchschnittlich etwa 3 Wochen unter typischen Symptomen. Als Kontrollmaterial diente das Z.N.S. von 12 gesunden Kaninchen (darunter 7 Wildkaninchen). Die Fixation von Teilen des Gehirnes und Rückenmarkes geschah unmittelbar nach dem Tode der Tiere in Bromformol, Formalin und teilweise in Alkohol. Von allen Fällen wurden zum Zwecke eines einwandfreien Vergleiches dieselben Hirnteile, nämlich Mittelhirn, Großhirnrinde, verlängertes Mark nach 1–3 tägiger Härtung auf dem Gefriermikrotom geschnitten und die Schnitte der Hortegaschen Silbercarbonatmethode unterworfen. In einem Teile der Fälle kam sie zum Nachweis von Fett und Eisen mit der Scharlachrotfärbung nach

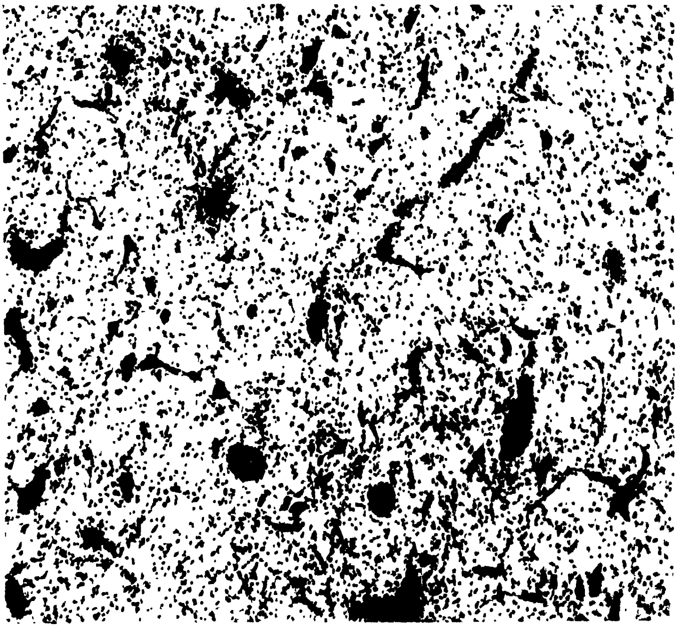

ABB. 1. Bornasche Krankheit beim Pferde. Herdförmige und diffuse Gliawucherung in der Substantia nigra. *Nissl*-Färbung. Vergr. 80mal.

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* Der Virusstamm wurde früher von Prof. *Zwick*, Gießen, dem Rockefeller-Institut in New York zur Verfügung gestellt, von wo er mir durch Dr. *Olitzky* gütigst überlassen wurde.

Herxheimer sowie mit der Turnbullblaumethode vereinigt zur Anwendung. Ihr Gelingen ist von vielen Zufälligkeiten abhängig. Es empfiehlt sich deshalb, mit peinlicher Genauigkeit die Vorschriften zu beachten (*Metz* und *Spatz*[7]; *Spielmeyer*sche Technik der mikroskopischen Untersuchung des Nervensystems[9]). Einzelheiten über den Chemismus, die färberischen Eigenschaften und die Herstellung ammoniakalischer Silberlösungen finden sich in einer neuen Arbeit von *Kubie* und *Davidson*[10]. Nach eigenen Erfahrungen ist die Verwendung doppeltdestillierten Wassers, reiner Gefäße und die richtige Dauer der Fixierung (1–2½ Tage) von besonderer Wichtigkeit. Für die Darstellung der Astrocyten wurde außerdem die *Cajal*sche Goldsublimatmethode und zur Darstellung der Neurofibrillen die *Bielschowsky*sche Färbung in Verbindung mit der *Herxheimer*schen Scharlachrotfärbung angewendet. Zur Ergänzung der mit diesen Methoden erhobenen Befunde wurden in verschiedenen Fällen gewöhnliche Färbungen (*Nissl*, Hämatox.-Eosin, *van Gieson*) herangezogen.

A. Morphologie und Verteilung der Hortegaschen Zellen unter normalen Verhältnissen

Bei diesen Voruntersuchungen habe ich mich auf die oben erwähnten Gehirnabschnitte beschränkt, weil in ihnen bei Bornascher Krankheit ausgesprochene Veränderungen auftreten [siehe *Seifried* und *Spatz*, Z. Neurol. **124**, 317 (1930)].

In gelungenen Präparaten sind die *Hortega*schen Zellen besonders in ihren Umrissen dunkel imprägniert, während die dickeren Hauptäste bei linienartiger Imprägnation ihrer Kontur mehr einen grauen Farbton annehmen. Im heller gefärbten Teil der Zellen treten ziemlich regelmäßig rundliche, mehr oder weniger zahlreiche, argentophile Körnchen, auf (siehe Abb. 2 und 3). Der Zellkern, der nie rund, sondern oval, eckig, stäbchen- oder biskuitförmig und bisweilen winkelig abgeknickt ist, färbt sich im allgemeinen dunkel und läßt zahlreiche Innenkörperchen hervortreten (siehe Abb. 2 und 3). Nur in seltenen Fällen ist er heller als das perinucleäre Protoplasma. Die übrige Gestalt, die Art der Verzweigung, das Auftreten bipolarer (siehe Abb. 2) oder multipolarer Typen (siehe Abb. 3), die stachelartigen Endfortsätze, die baumastartigen Abknickungen sind zwar veränderlich, in ihrer Form aber stets charakteristisch. Es gehört zu ihrer besonderen Eigenart, daß sie unter normalen Verhältnissen außerordentlich klein sein können (siehe Abb. 4). Mitosen an ihnen werden nicht beobachtet; dagegen sind Bilder, die an direkte Teilungen erinnern, nicht selten.

Bereits normalerweise können die *Hortega*schen Zellen Lipoide in mäßigen Mengen im perinucleären Protoplasma und in den Verzweigungen enthalten. Dagegen wird die Speicherung von Eisenpigment nur ausnahmsweise und in geringen Mengen beobachtet. Ob die *Hortega*schen Zellen ein zusammenhängendes Netz darstellen, läßt sich nicht feststellen. Im allgemeinen erscheinen die Zellen als Einzelindividuen

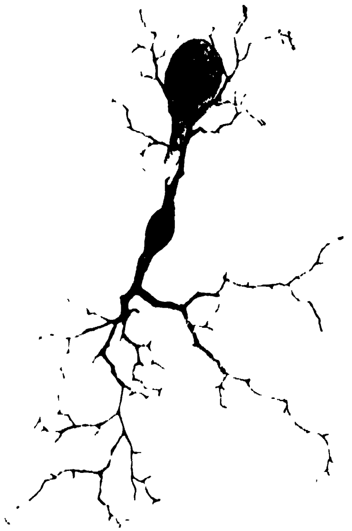

Abb. 2

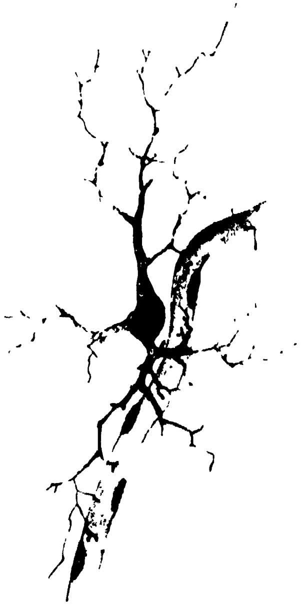

Abb. 3

Abb. 2. Normales Kaninchen. Bipolarer Typus einer *Hortega*schen Zelle in Verbindung mit einer Nervenzelle Mittelhirn. *Hortega*sche Methode.

Abb. 3. Experimentelle Bornasche Krankheit beim Kaninchen (gestorben 10 Tage nach der Impfung). Leicht hypertrophische *Hortega*sche Zelle (multipolarer Typus) in Begleitung eines Gefäßes. Mittelhirn. *Hortega*sche Methode.

(siehe Abb. 4); Verbindungen von Fortsätzen benachbarter Zellen können nur selten festgestellt werden.

Das Studium der *Verteilung der Hortegaschen Zellen* in einem bestimmten Hirnabschnitt wird erleichtert, wenn gut gelungene Färbungen d. h. solche ohne wesentliche Mitfärbung anderer Elemente benutzt werden. Am ehesten erhält man solche nach 24–48 stündiger Fixier-

ung dünner Gehirnstücke in Bromformol. Aber selbst bei Einhaltung dieser Zeit und bei Beachtung aller Sorgfalt bei der Herstellung der Lösungen, werden häufig aus unbekannten Gründen weniger günstige Ausfälle der Methode erzielt. Vielfach begegnet man nicht nur einer stärkeren Imprägnation von Nervenzellen, sondern auch von faserbildenden Astrocyten, die sich durch ihren großen, runden und hellen Kern, ihre dicken, bisweilen geschlängelten, meistens unverzweigten Fortsätze und ihre haftscheibenförmigen Endungen an Gefäßwänden ohne weiteres von *Hortega*schen Zellen unterscheiden lassen (siehe Abb. 5). Auch die Oligodendro-Gliazellen sind an ihrer epithelioiden Gestalt leicht zu erkennen, während protoplasmatische Astrocyten weniger deutlich hervortreten. Aber auch derartige Präparate werden—wie auch *Metz* und *Spatz* erwähnen—mit Vorteil herange-

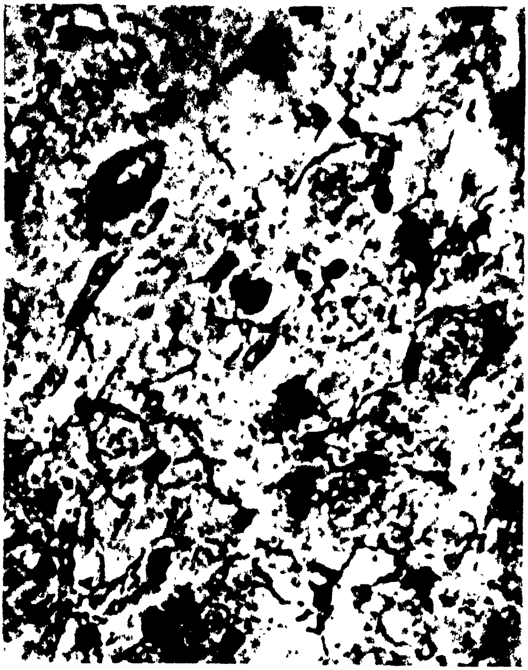

Abb. 4. Normales Kaninchen. *Hortega*sche Zellen. Mittelhirn. *Hortega*sche Methode. Vergr. 530mal.

zogen, weil sie die Lagebeziehungen der *Hortega*schen Zellen zu anderen Elementen deutlich veranschaulichen. Im allgemeinen zeigen sie eine ziemlich gleichmäßige Verteilung; sie treten häufig in Zusammenhang mit Nervenzellen (siehe Abb. 2), mit anderen Gliaelementen sowie mit Gefäßen (siehe Abb. 3) auf. Selbst die Begleitzellen der Ganglienzellen sind vielfach *Hortega*sche Elemente. Ihre Zahl ist verhältnismäßig spärlich; sie scheint in verschiedenen Fällen und auch in verschiedenen Hirnabschnitten gewissen Schwankungen unterworfen zu sein. Aber selbst bei reichlicherem Vorkommen gewinnt man nie den Eindruck einer dichten Lagerung oder gar von Ansammlungen.

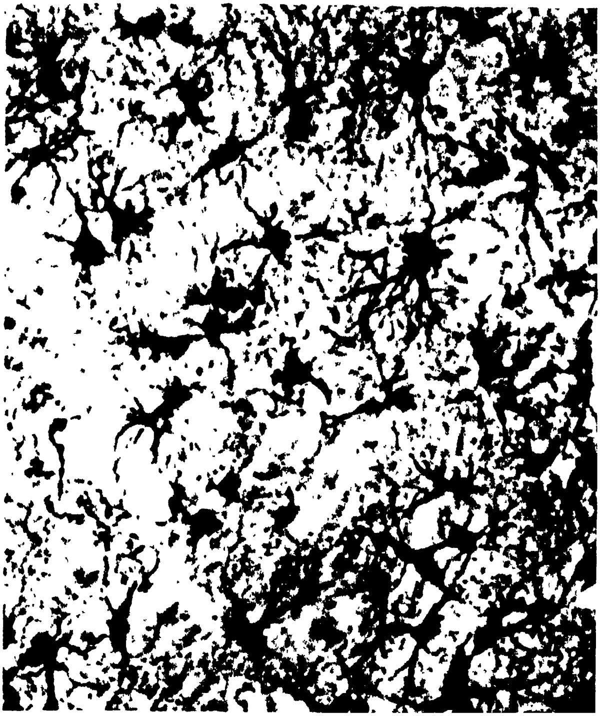

Abb. 5. Experimentelle Bornasche Krankheit beim Kaninchen (gestorben 10 Tage nach der Impfung). Typische Astrocyten. Mittelhirn. *Cajal*sche Goldsublimatmethode. Vergr. 460mal.

B. Morphologie und Beteiligung der Hortegaschen Zellen im entzündlichen Reaktionskomplex der Bornaschen Krankheit

Im Gehirne von Kaninchen, die zwischen 3 und 10 Tagen nach der intracerebralen Infektion mit Bornavirus getötet worden waren, wird bereits eine Vermehrung der *Hortega*schen Zellen beobachtet. Wenn nicht zum Vergleiche eine Reihe von Normalfällen herangezogen wird, ist die zahlenmäßige Zunahme der Zellen oft nicht auffallend, weil sie in ziemlich gleichmäßiger Verteilung vorhanden sind. Gliaknötchen oder dichtere Gliaproliferationen werden in diesen Frühstadien noch vermißt. Bisweilen gewinnt man den Eindruck, als ob die Elemente in unmittelbarer Nachbarschaft von Gefäßen eine besondere Zunahme

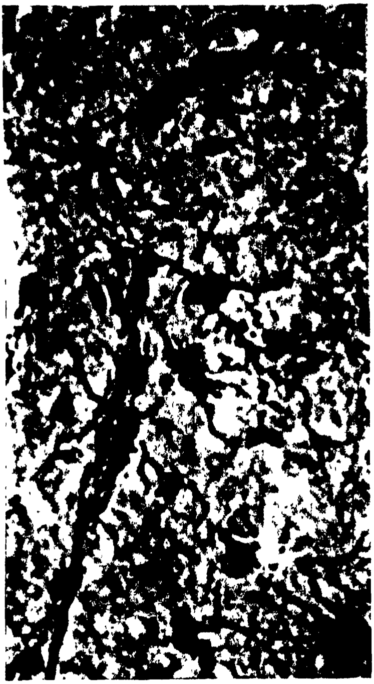

Abb. 6. Experimentelle Bornasche Krankheit beim Kaninchen (gestorben 10 Tage nach der Impfung). Vermehrung und leichte Hypertrophie *Hortega*scher Zellen im Bereiche eines kleinen Gefäßes. Hirnrinde. *Hortega*sche Methode. Vergr. 740mal.

erführen (siehe Abb. 6), und zwar ehe es zur Bildung von vasculären oder perivasculären Infiltraten kommt. Man sieht hier oft außerordentlich kleine Elemente, die als Jugendformen der *Hortega*schen Zellen zu gelten haben. Diese frühe Beteiligung der *Hortega*schen Zellen im entzündlichen Reaktionskomplex der Bornaschen Krankheit ist eine Eigenschaft, die der Neuroglia nicht in gleichem Maße zukommt. Bilder, die an direkte Teilungsformen erinnern, sind in diesen Stadien häufiger als in Normalfällen.

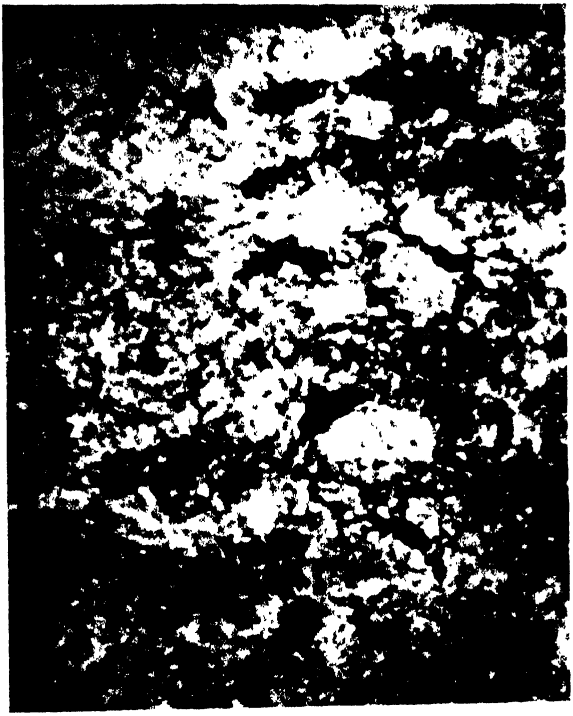

Abb. 7. Experimentelle Bornasche Krankheit beim Kaninchen (Spätstadium: gestorben 35 Tage nach der Impfung). Hypertrophie und Umwandlungsstadien *Hortega*scher Zellen. Mittelhirn. *Hortega*sche Methode. Vergr. 530mal.

Viel auffallender als die zahlenmäßige, diffuse Vermehrung der *Hortega*schen Zellen bei der Bornaschen Krankheit, sind ihre morphologischen Veränderungen, die damit einhergehen. Die einzelnen

Zellen zeigen neben einer auffallenden Hypertrophie (vgl. Abb. 4 und 7, mit derselben Vergrößerung aufgenommen) wesentliche Änderungen ihrer äußeren Gestalt. Ihre Eigentümlichkeiten treten mit größerer Deutlichkeit hervor, nicht nur weil die normalerweise so feinen Fortsätze verbreitert, ja sogar plump sein können, sondern weil auch ihre Konturen stärker imprägniert sind (siehe Abb. 6, 7, 8, 9). Schon bei leichter Hypertrophie der Zellen sieht man ihre Eigenschaft,

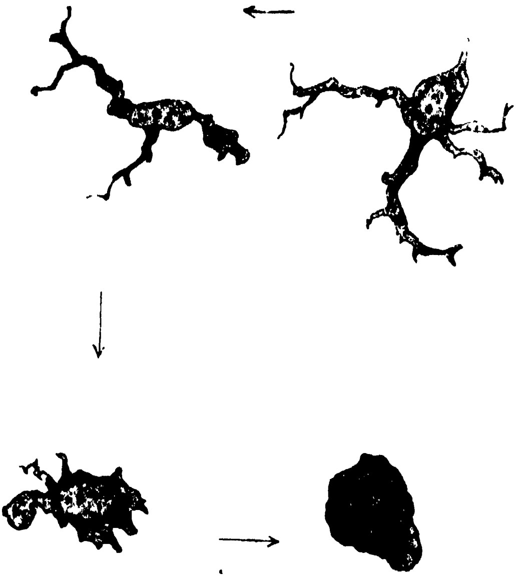

Abb. 8. Experimentelle Bornasche Krankheit beim Kaninchen (Spätstadium: gestorben 35 Tage nach der Impfung). *Hortega*sche Zellen in verschiedenen Stadien der Umwandlung zur Gitterzelle. Mittelhirn. *Hortega*sche Methode.

Silber anzuziehen, stärker betont; in späteren Stadien ist sie geradezu ein Merkmal pathologisch veränderter Zellen. Auch die Kerne derartig hypertrophischer Zellen sind vergrößert, unregelmäßig geformt, häufig stäbchen- oder wurstförmig und manchmal abgeknickt. Bipolare Typen (Stäbchenzellen) sind besonders häufig, aber auch multipolare kommen vor. In älteren Fällen sieht man oft Typen, die, obwohl sie von ihrer ursprünglichen Form weit abweichen, doch immer noch charakteristische Merkmale genug besitzen, daß sie von anderen gliösen Elementen ohne große Schwierigkeit unterschieden werden können. Sie sind ihrer feineren Verästelung völlig verlustig gegangen; die wenigen noch vorhandenen Fortsätze werden kürzer und plumper, an den Enden abgerundet und oft keulenförmig verdickt (siehe Abb. 7, 8, 9). Gleichzeitig nehmen die Zellen eine rundliche Gestalt an (siehe Abb. 7 und 8); demnächst sieht man nur noch kurze, stachelförmige Fortsätze an ihrer Peripherie (siehe Abb. 7). Die Fähigkeit der Umwandlung derartig veränderter Hortegascher Zellen in Fettkörnchenzellen läßt sich oft unter Aufweisung lückenloser Zwischenstadien verfolgen (siehe Abb. 7 und 8). Auch die Speicherung von Fettsubstanzen in derartigen Zellen kann deutlich dargestellt werden (siehe Abb. 9), während eine abnorme oder gar elektive Eisenspeicherung nicht vorkommt.

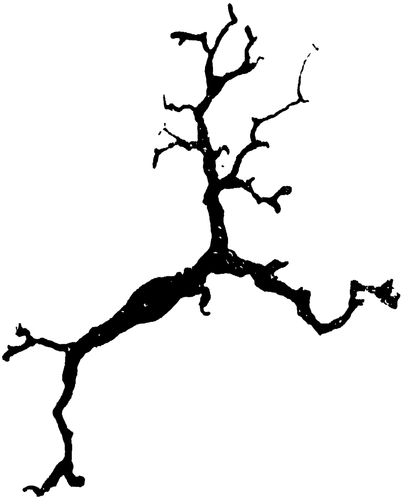

Abb. 9. Experimentelle Bornasche Krankheit beim Kaninchen (gestorben 25 Tage nach der Impfung). Hypertrophische, mit Fettröpfchen beladene *Hortegasche* Zelle. Hirnrinde. Kombination der *Hortega*schen Methode mit *Herxheimers* Scharlachrotfärbung.

Abgesehen davon, daß die Fähigkeit der Umwandlung in Gitterzellen nicht eine besondere Eigenart der Hortegaschen Zellen ist, wie *Gallego*, *Bratianu* und *Llombard* u. a. annehmen, sondern daß gewöhnliche Gliazellen und Gefäßwandzellen dieselbe Eigenschaft besitzen, ist das Vorkommen von Körnchenzellen nicht etwa ein besonders hervorstechendes Merkmal im histologischen Bilde der Bornaschen Krankheit. Sie sind im Gegenteil sehr selten, worauf auch bereits *Dobberstein* hingewiesen hat. Ihr Auftreten ist wahrscheinlich ganz von der jeweiligen Anwesenheit von Abräumstoffen abhängig, die in späteren Stadien mit Degeneration von Neurofibrillen und Ganglienzellen häufiger zugegen sind als in früheren. Die spezifisch funktionelle Bedeutung der Hortegaschen Zellen darf nicht überschätzt werden. Ihre Hypertrophie ist auch keineswegs immer auf die Aufnahme phagocytierbarer Substanzen zurückzuführen, sondern sie ist offensichtlich häufig das Vorstadium degenerativer Vorgänge. Formen mit Verlust der Fortsätze, Zerfall des Zellkerns, ohne sichtbare Zeichen einer phagocytären Tätigkeit, können kaum anders als Degenerationserscheinungen gedeutet werden. Die erhöhte Argentophilie derartiger Formen und die häufig beobachteten Degenerationen der Neuroglia, lassen sich mit einer derartigen Deutung durchaus in Einklang bringen.

Es erhebt sich nun vor allem die Frage, in welchem Umfange die Hortegaschen Zellen im Bereiche von perivasculären Infiltraten vorkommen und inwieweit sie an der Bildung der bekannten Gliaknötchen, der mehr diffusen Gliaherde sowie an den Prozessen der sog. „Umklammerung" beteiligt sind.

Was die *perivasculären Infiltrate* anbetrifft, so bestehen sie im wesentlichen aus mononucleären Elementen. *Hortega*sche Zellen sind an ihrem Aufbau unmittelbar nicht beteiligt, dagegen findet man an ihrem Rande oder in ihrer unmittelbaren Nachbarschaft nicht selten auch proliferativ veränderte *Hortega*sche Zellen in geringer Zahl (siehe Abb. 10). Diese Befunde decken sich mit denjenigen, die bei Anwendung gewöhnlicher Methoden erhoben werden. Das Auftreten von Stäbchenzellen, einer besonderen Form proliferativ veränderten *Hortega*scher Elemente, am Rande von perivasculären Infiltraten ist in nach *Nissl* gefärbten Präparaten keine Seltenheit. Daß gleichzeitig in der Umgebung von Gefäßinfiltraten auch eine Vermehrung von Astrocyten (oft in kranzförmiger Anordnung) stattfindet, kann bei Anwendung der *Cajal*schen Methode häufig gezeigt werden (siehe Abb. 10).

In den feineren Aufbau der *Gliaknötchen* läßt sich ebenfalls schon in frühen Stadien der Krankheit Einblick gewinnen. Häufig sieht man kleine, aus 5-8 Zellen bestehende Anhäufungen, die vollkommen aus proliferativ veränderten *Hortega*schen Zellen zusammengesetzt sind. Auch *größere Zellansammlungen* können lediglich aus *Hortega*schen Elementen aufgebaut sein (siehe Abb. 11). Sie tragen im einzelnen die beschriebenen Merkmale proliferativ und degenerativ veränderter Typen. Auf der anderen Seite läßt sich feststellen, daß die knötchenförmigen Herde vielfach eine gemischte Zusammensetzung besitzen, d. h. daß neben *Hortega*schen Zellen verschiedene Formen der Neuroglia, ja sogar mononucleäre Zellen wesentlich beteiligt sind. Dasselbe trifft für die mehr diffusen Gliaherde zu, die selten aus reinen *Hortega*schen Zellen zusammengesetzt sind. Im allgemeinen erreichen die verschiedenen Formen der Gliaproliferation bei der experimentellen Krankheit des Kaninchens nicht den Grad wie bei der Spontankrankheit des Pferdes.

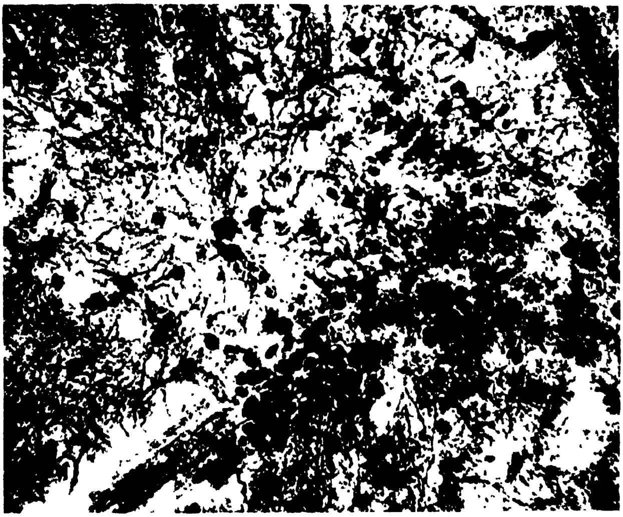

Abb. 10. Experimentelle Bornasche Krankheit beim Kaninchen (gestorben 15 Tage nach der Impfung). Hypertrophische *Hortega*sche Zellen am Rande eines perivasculären Rundzelleninfiltrates. Mittelhirn. *Hortega*sche Methode. Vergr. 310mal.

Die *die Ganglienzellen begleitenden Elemente* sind hauptsächlich Oligodendrogliazellen. Aber auch *Hortega*sche Zellen können als

Satteliten auftreten und die an den *neuronophagischen* Prozessen beteiligten Zellen werden ebenfalls, besonders an ihren Randpartien, teilweise durch *Hortega*sche Zellen dargestellt. Auch diese Befunde werden durch den Nachweis von typischen Stäbchenzellen im Bereiche solcher Bildungen bei Anwendung der Nisslfärbung bestätigt.

Zum Schluß muß noch betont werden, daß oft in älteren Stadien der Bornaschen Krankheit die Vermehrung der *Hortega*schen Zellen weniger auffallend ist als in jungen, und daß die Hypertrophie und Formumwandlung der Zellen die hauptsächlichsten Veränderungen darstellen können.

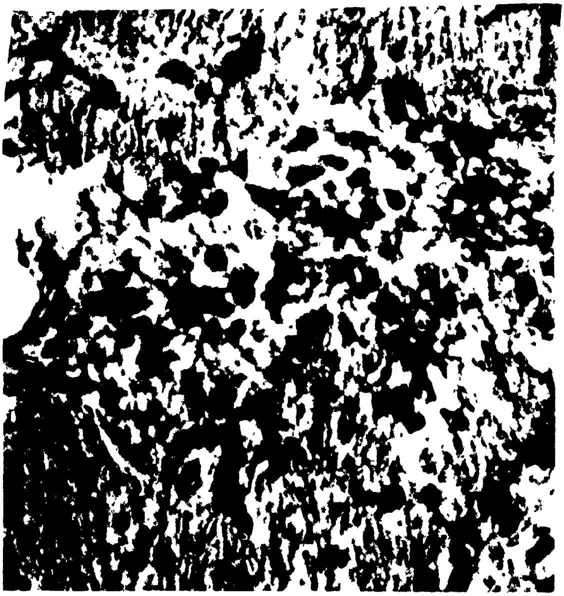

ABB. 11 Experimentelle Bornasche Krankheit beim Kaninchen (gestorben 15 Tage nach der Impfung). Zellherd fast ausschließlich aus proliferativ veränderten *Hortega*schen Zellen zusammengesetzt. Mittelhirn. *Hortega*sche Methode. Vergr. 740mal.

ZUSAMMENFASSUNG UND SCHLUẞ

1. Bereits vor dem Auftreten vasculärer und perivasculärer Infiltrate und in späteren Stadien der experimentellen Bornaschen Krankheit beim

Kaninchen wird eine Vermehrung der Hortegaschen Elemente beobachtet. Gleichzeitig damit geht eine Hypertrophie der Zellen, verbunden mit einer wesentlichen Formveränderung und erhöhter Argentophilie einher. Sowohl bipolare (Stäbchenzellen) und multipolare Formen kommen vor. In älteren Fällen kann die Hypertrophie der Hortegaschen Zellen im Vordergrunde stehen, während ihre Vermehrung nicht so auffällig ist.

2. Auch bei der Bornaschen Krankheit können die Hortegaschen Zellen vermehrt lipoide Stoffe aufnehmen und sich unter Verlust ihrer Verästelung und ihrer Fortsätze in Gitterzellen umwandeln. Dieser Prozeß spielt indessen eine untergeordnete Rolle. Degenerative Veränderungen an den Hortegaschen Zellen kommen ebenfalls vor.

3. Ähnlich wie bei anderen neurotropen Viruskrankheiten nehmen proliferativ veränderte Hortegasche Zellen entweder allein oder neben neuroglialen Elementen an der Bildung von Gliaknötchen und Gliaherden teil. Auch am Rande von perivasculären Rundzelleninfiltraten können sie, ebenso wie die Astrocyten, vorkommen. Außerdem sind sie an dem Prozeß der „Umklammerung“ beteiligt. Sie stellen einen wesentlichen Faktor im „entzündlichen Reaktionskomplex“ der Bornaschen Krankheit dar und müssen, zusammen mit den Veränderungen der Neuroglia, den vasculären und perivasculären Infiltraten gleichwertig an die Seite gestellt werden.

SCHRIFTTUM

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AN EXPERIMENTAL STUDY OF "MAD ITCH" WITH ESPECIAL REFERENCE TO ITS RELATIONSHIP TO PSEUDORABIES

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INTRODUCTION

In August of 1930 an outbreak of "mad itch" was observed in a herd of dairy cattle in Johnson County, Iowa.[1] In order to evaluate the experimental disease, an account of the clinical picture as observed in this herd will be given. The disease is so rare as to constitute a veterinary curiosity.

There were twelve animals in the herd and of these nine succumbed to the disease. No evidence of illness was observed in the remaining three. The clinical course in the nine fatal cases was rapid and in all but one an extreme pruritus was the cardinal feature of the disease. A persistent and continuous licking of an area of skin somewhere on the hind quarters, most commonly between the udder and leg, and a sudden decrease in milk production constituted the first evidence of illness. After 2 or 3 hours of continual licking the area of skin would be completely denuded of hair and bright red in color. The condition was steadily progressive and the area of abraded skin became larger. The pruritus evidently increased in intensity for the animals would rub violently against posts, barbed wire, gates, or any other convenient object, and would bite and gnaw frenziedly at themselves. By the end of 24 hours the buttocks and thighs were denuded, swollen and discolored, oozing a serosanguineous fluid, and the animals were usually down although still able to rise and walk unsteadily about. There was no temperature elevation at this time. Salivation and marked grinding of the teeth were observed in some cases. The animals became progressively weaker and died within 36 to 48 hours after the first evidence of illness. Death was preceded by clonic convulsions, violent tossing of the head, crying, and rapid shallow respiration. One of the nine animals had failed to show any evidence of pruritus. This animal was ill for 6 days and lay listlessly in the barn lot during this period. On the last day she developed clonic convulsions, salivated, ground her teeth, and died in much the same manner as the others.

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[1] Dr. Fred J. Crow of Iowa City called my attention to this outbreak of "mad itch," made the material available to me, and cooperated in the preliminary field work. I thank him heartily.

Attempts to Reproduce "Mad Itch" Experimentally

Cerebrospinal fluid in a sterile container from one cow and portions of brain from three others in 50 per cent glycerine were brought back to the laboratory on ice. A period of 2 weeks elapsed between the time of the collection of this material and its inoculation. Samples of various specimens were injected subcutaneously and intracerebrally into rabbits, guinea pigs, rats, and mice. Only one was found capable of inducing recognizable disease, this being the brain from the cow that had failed to exhibit any evidence of pruritus and had survived longer than any of the other affected animals.

Physiological salt solution suspensions of the effective brain when injected subcutaneously into rabbits reproduced the clinical picture of "mad itch" as seen in the cattle.

After incubation periods usually ranging from 50 to 75 hours the rabbits appeared uneasy and began biting and scratching mildly at the site of inoculation. There was no temperature elevation at this time. The condition became progressively worse and the biting and scratching more persistent and savage. The skin over the site of inoculation and over a large surrounding area was soon denuded of hair and abraded, and it oozed a serosanguineous fluid. As the animals became weaker, their attempts at biting and scratching were more feeble. They lay on their sides unable to rise, their respirations became rapid and in some cases noisy, and as a rule there was a marked temperature elevation. Death ensued from 6 to 24 hours after the first evidence of pruritus and it was preceded by loss of consciousness and mild clonic convulsions.

This clinical picture has been produced regularly and in quite a large series of rabbits by the subcutaneous injection of either suspensions of the original cow brain or of brains of intracerebrally infected rabbits or guinea pigs. Four out of five attempts to infect guinea pigs by subcutaneous inoculation with the original cow brain were unsuccessful and the material was also innocuous when administered subcutaneously to white rats and white mice.

Injected intracerebrally, the material produced a uniformly fatal disease in rabbits, guinea pigs, and white rats and mice.

Rabbits died in from 24 to 50 hours following inoculation and the period of actual illness was short. After an incubation period of from 20 to 40 hours the animals appeared nervous and excited and soon were observed to be blind. They became more excited and would sometimes run wildly about their cages butting their heads violently against the door. At other times they would retract their heads, lifting a front foot as though fearing attack. They frequently continued the retraction of their heads until they would rise on their hind legs and fall over backwards. No temperature elevation was to be observed until just before death when a marked rise occurred. Death was usually preceded by coma and rapid and sometimes noisy respiration. Salivation and grinding of the teeth were fairly constantly to be observed.

The above picture has been produced regularly in rabbits by the intracerebral injection of the original cow brain or by the injection of suspensions of brains from intracerebrally infected rabbits or guinea pigs. No pruritus resulted following intracerebral inoculation. The symptoms observed in guinea pigs, rats, and mice following intracerebral inoculation were similar to those seen in rabbits but they were of less violent character and the periods of incubation and survival were greater.

The disease induced in rabbits by the intranasal instillation of "mad itch" virus deserves special mention in that it differed from that induced by cerebral or subcutaneous inoculation.

For a period of 4 days following infection the animals appeared perfectly normal, ate well, and exhibited no temperature elevation. On the beginning of the 5th day the respiration of some of the animals was slightly accelerated and in one frequent sneezing was observed. The animals did not appear ill, however, and there was no temperature elevation. Death occurred suddenly on the 5th day and without warning. At autopsy the most significant pathology was found in the lungs. These were of a glistening purplish red color, heavy, and filled with a serosanguineous fluid. The bronchi and trachea usually contained an abundance of a blood-tinged frothy exudate and there were many submucous hemorrhages.

Both the clinical and the postmortem picture of the disease produced by the intranasal instillation of the virus are strikingly suggestive of a rather common bovine disease which, like "mad itch," is generally classed as hemorrhagic septicemia.

Gross Pathology

The postmortem picture of cattle dead of the spontaneous disease was always the same. The skin over the thighs and buttocks was denuded of hair, dark, leather-like, and smeared with a serosanguineous fluid. When the skin in these regions was incised a dark red, serous, and occasionally gelatinous material was encountered in the greatly thickened subcutaneous tissue. The underlying muscle was not involved. The abdominal viscera, aside from a moderate congestion, appeared normal. The stomachs were well filled. The lungs of some of the cows exhibited areas of an edematous type of congestion; the hearts of all were spotted with numerous petechial hemorrhages, and in all cases there was an excess of pericardial fluid. Aside from moderate injection of the meningeal vessels nothing pathological in the gross was encountered in the central nervous system. There was thus no gross anatomical cause of death apparent in the spontaneous cases.

In rabbits inoculated subcutaneously, the skin in the region of the site of inoculation was denuded of hair, abraded, and moistened by a serosanguineous fluid. The underlying subcutaneous tissue was thickened, edematous, and bloody. The regional lymph nodes were enlarged and congested. The stomach, intestines, and colon were found to be well filled. The urinary bladder was, as a rule, greatly distended. The liver was strikingly of a "nutmeg" appearance. The spleen and kidneys were at the most no more than moderately congested. The heart was injected and at times spotted with petechial hemorrhages. The thymus was large, sometimes almost completely covering the heart, and was dotted with variously sized areas of hemorrhage. The lungs were a glistening purplish red, heavy, and on cut section exuded a serosanguineous fluid. The bronchi often contained a thin frothy mucus. Aside from a mild to moderate injection of the meningeal vessels nothing abnormal was encountered in the central nervous system. In contrast to the absence of gross lesions in cattle, the intensely hemorrhagic pulmonary edema could be readily held accountable for the death of experimentally infected rabbits. The gross pathological picture presented by intracerebrally inoculated rabbits was the same as that outlined for subcutaneously inoculated animals except that the lesions of the skin and subcutaneous tissue were absent. The postmortem picture presented by guinea pigs dead of experimental "mad itch" was the same as that seen in rabbits except that the lesions in the skin and subcutaneous tissue were more extreme and the pulmonary lesions less marked.

The histopathology will be reported later.

Etiology

As has been reported briefly elsewhere (1), no ordinary bacterial organism has been discovered in "mad itch" materials of proven infectivity, by methods of culture and direct microscopic examination. On the other hand, suspensions of emulsified brain of intracerebrally inoculated rabbits when passed through Chamberland L_3 and Berke-

TABLE I

Filtration Experiments

Filter		Material filtered, approximately 10% suspension of rabbit brain		Time of filtration	Amount of filtrate	Maximum negative pressure	Dosage of filtrate administered and route	Rabbit inoculated. No.	Result
Type	No.	Suspension	Rabbit No.						
				min.	*cc.*	*cm. of mercury*	*cc.*		
1. Seitz		A	84 and 85	2		Positive pressure	1 sc.*	95	No illness
							0.15 ic.	76	" "
2. "		B	76, 94, and 95	2	20	" "	1 sc.	114	Died—105 hrs.
3. "		B	76, 94, and 95	2	25	" "	1 sc.	108	No illness
4. Berkefeld V	10	A	84 and 85	0.5		28	1 sc.	92	Died—96 hrs.
5. " V	10	B	76, 94, and 95	1	26	48	1 sc.	106	" —76 "
6. " N	6	A	84 and 85	0.5		37	1 sc.	90	" —99 "
							0.15 ic.	75	" —46 "
7. " N	1	B	76, 94, and 95	2	20	49	1 sc.	107	" —76 "
8. " N	7	B	76, 94, and 95	2	10	30	1 sc.	111	" —90 "
9. " N	8	B	76, 94, and 95	2	20	57	1 sc.	105	" —98 "
10. " N	1	C	110	3	40	45	1 sc.	131	" —89 "
11. " W	9	B	76, 94, and 95	2	6	42	1 sc.	110	No illness
12. " W	11	B	76, 94, and 95	2	8	60	1 sc.	109	" "
13. " W	11	D	108	12	5.5	60	0.15 ic.	109	Died—50 hrs.
14. " W	11	C	110	2	3	43	1.5 sc.	126	No illness
15. " W	11	C	110	12	10	60	1.5 sc.	125	Died—89 hrs.
16. Chamberland L^3	1	B	76, 94, and 95	2	5	55	1 sc.	112	" —125 "
17. " L^3	2	B	76, 94, and 95	2	3.5	45	1 sc.	113	No illness
18. " L^3	4	C	110	10	10	63	1.5 sc.	129	Died—101 hrs.

* sc. = subcutaneously.
ic. = intracerebrally.

feld V, N, and W filters were effective in inducing the disease in rabbits. A summary of the results is given in Table I.

In all but one of the filtrations (No. 7) infusion broth pH 7.3 was used as the vehicle in preparing the brain suspensions, but in this one case in which physiological salt solution was used no observable difference in the filtrability could be noted. In some of the experiments the brain suspensions were frozen and thawed before filtration. This procedure was unessential, for unfrozen suspensions filtered with equal facility. All suspensions were cleared of gross particles by centrifugation for 30 minutes before filtration. The moderately turbid supernatant fluid was then removed for use in the filtrations. To this was added 24 hour broth cultures of *B. prodigiosus* in the ratio of 1 cc. of culture to each 10 to 15 cc. of suspension to be filtered. 1.5 cc. amounts of the resulting filtrates were tested for sterility, as regards *B. prodigiosus*, in broth culture. All filtrates recorded in the protocol except Seitz Filtrates 2 and 3 were bacteriologically sterile. As can be seen from the table, time was an important factor in the passage of the virus through the finer pored filters. Out of five attempts at filtration through Berkefeld W filters, three filtrates in which the filtration was continued for only 2 minutes did not contain virus demonstrable in the dosage employed, while two filtrates in which the filtration was continued for 12 minutes contained quantities of virus lethal for rabbits in ordinary dosage. Difficulty was experienced in the Seitz filtrations because of the facility with which these filters passed *B. prodigiosus* in infusion broth. In one Seitz filtration in which *B. prodigiosus* was retained by the filter the "mad itch" virus was not present in demonstrable quantities in the filtrate. Two other Seitz filtrations are difficult to interpret because while both permitted the passage of *B. prodigiosus* only one permitted the passage of virus in demonstrable quantity. Animals inoculated with filtered material were adequately isolated from animals receiving unfiltered material.

The recording of the same animal in more than one experiment requires explanation. Rabbits negative in certain experiments have, after a period of observation of 2 weeks, been used over again in later experiments. The procedure may be questioned because of the possible development of some immunity following the administration of sublethal doses of virus. However, in the experiments recorded in Table I no animal, negative in an earlier experiment, failed to develop "mad itch" and die when later inoculated. This eliminates from consideration the possibility that in these experiments a confusing immunity had been set up as a result of the earlier use of the animals.

Neutralization of Virus with Immune Serum

It has been possible to induce an immunity in a guinea pig sufficient to protect that animal completely against a subcutaneous dose of rabbit brain virus always lethal for unprotected guinea pigs.

The procedure, in brief, consisted in the preliminary subcutaneous administration of the original cow brain virus which was capable of producing a fatal infection in only one out of five guinea pigs to which it was administered subcutaneously. The four guinea pigs that had been refractory to inoculation with cow brain virus were subsequently inoculated subcutaneously with rabbit brain virus known to be capable of inducing a fatal infection when administered subcutaneously to guinea pigs. Three of the animals died and the fourth (Guinea Pig 168) exhibited no evidence of illness. This fourth guinea pig has since been inoculated subcutaneously seven times with various suspensions of rabbit brain virus of proven pathogenicity for guinea pigs, and has shown no evidence of illness following any of the inoculations. The animal has been bled repeatedly from the heart at suitable intervals between inoculations with virus and the serum has been tested for its ability to neutralize virus. Typical experiments are recorded in Table II.

In the virus neutralization experiments the mixtures were usually kept at room temperature for 2 hours and at refrigerator temperature overnight before inoculation into the test animals. This preliminary period of incubation was unnecessary, for virus mixed with immune serum just before injection, into guinea pigs, was apparently as completely neutralized. Neither was it necessary to have a relatively cell-free virus suspension to attain complete neutralization, for in a number of instances the coarse uncentrifuged brain suspensions were completely neutralized by small amounts of immune serum. With the use of smaller doses of virus and larger amounts of immune serum than are recorded in Table II, it has recently been possible to attain mixtures that were completely neutral for rabbits. It has also been found that mixtures neutral for guinea pigs when administered subcutaneously are also neutral when injected intraperitoneally.

Routes of Infection

Uniformly fatal infections in rabbits have resulted following subcutaneous, intracerebral, intravenous, intratesticular, intraperitoneal, and intranasal inoculation with the "mad itch" virus. Fatal infections result from small doses of virus (0.2 cc.) injected into the tip of the ear and also from scarification through a drop of virus placed on a shaven area of skin. 0.001 cc. of the supernatant fluid of a centrifuged 10 per cent suspension of rabbit brain virus was sufficient to induce typical fatal "mad itch" in a rabbit when injected subcutaneously, whereas 0.0001 of the same virus suspension failed to infect.

TABLE II

Neutralization Tests with Immune Guinea Pig Sera

Source of virus	Amount of virus	Serum	Animal*	Result
	cc.			
			Rabbit	
Supernatant fluid of centrifuged suspension of brains of Rabbits 87, 96, and 97	1	1 cc. immune serum Guinea Pig 168	99	Died—122 hrs.
	1	0.5 cc. immune serum Guinea Pig 168	100	" —112 "
	1	0.1 cc. immune serum Guinea Pig 168	101	" —103 "
	1	1 cc. normal serum Guinea Pig 211	102	" — 74 "—Control
Berkefeld N filtrate of suspension of brain of Rabbit 110	1	2 cc. immune serum Guinea Pig 168	128	Died—174 hrs.
	1	0.5 cc. immune serum Guinea Pig 168	130	" —113 "
	1	1.5 cc. normal serum Guinea Pig 211	131	" — 89 " — Control
			Guinea Pig	
Supernatant fluid of centrifuged suspension of brain of Rabbit 140	1	1 cc. immune serum Guinea Pig 168	235	No illness
	1	0.1 cc. immune serum Guinea Pig 168	234	" "
	1	1 cc. normal serum Guinea Pig 233	236	Died— 83 hrs. Control
10% suspension of brain of Rabbit 165	1	1 cc. immune serum Guinea Pig 168	263	No illness
	1	0.1 cc. immune serum Guinea Pig 168	262	" "
	1	1 cc. normal serum Guinea Pigs 233 and 238	261	Died— 45 hrs.—Control

* All animals inoculated subcutaneously.

Guinea pigs develop fatal infections uniformly following subcutaneous, intraperitoneal, intratesticular, intracerebral, or intranasal inoculation with rabbit brain "mad itch" virus.

White rats and mice develop fatal infections irregularly following intraperitoneal inoculation, regularly following intracerebral inoculation, and are refractory to subcutaneous inoculation with the "mad itch" virus.

Distribution of Virus in the Animal Body

The "mad itch" virus tends to localize largely in the region of the site of inoculation and the lung. The brain of an animal inoculated intracerebrally will be regularly rich in virus, and the lung will also contain it. However, virus cannot be demonstrated in the heart blood, liver, and spleen. In animals inoculated subcutaneously virus is demonstrable in the bloody fluid of the subcutaneous tissue at the site of inoculation and in the lung, but it has not been demonstrable in the heart blood or spleen. Neither is it demonstrable in the brains of subcutaneously inoculated animals unless these animals have survived unusually long as the result of partial protection by immune serum. It was present in the liver of one out of three animals examined. In animals infected intranasally the virus is found in both the lung and the brain but not in the liver or blood. As dependable sources of virus the brains of intracerebrally inoculated animals or the testicles of intratesticularly inoculated animals can be relied upon. In testing for the presence of virus in various tissues 1 cc. of a 10 per cent suspension was uniformly the inoculum employed.

Animals Susceptible to the "Mad Itch" Virus

While most of our experience has been with rabbits, guinea pigs, and white rats and mice, we have tested some other species for susceptibility to the "mad itch" virus. The virus, after fourteen serial intracerebral passages in rabbits was still capable of producing a fatal infection when injected subcutaneously into its natural animal host. A calf inoculated subcutaneously with 1.5 cc. of a 6.6 per cent suspension of rabbit brain virus, after an incubation period of 4 days developed typical "mad itch" simulating in all respects the spontaneous disease as seen in the original cows in Iowa. The animal died after about 45 hours of illness. The virus produced a fatal "itching" disease when inoculated subcutaneously into a cat. Gray field mice succumbed following intraperitoneal inoculation. Two ducks inoculated intracerebrally with the virus died after 4 and 18 days, and a suspension prepared from the brain of the first of these birds and inoculated in relatively small dosage subcutaneously into a rabbit resulted in a typical fatal "mad itch." One duck inoculated subcutaneously failed to develop any evidence of illness. The virus was innocuous for chickens when administered subcutaneously or intratracheally but following intracerebral inoculation fatal infections resulted in two out of three cases in 6 and 11 days. A suspension prepared from the brain of the first of these chickens and injected in relatively small dosage subcutaneously into a rabbit resulted in typical fatal "mad itch," proving the etiological nature of the disease to which the chickens had succumbed. Two swine inoculated subcutaneously with the virus became ill. No pruritus was observed in either animal. One animal developed what appeared to be an acute encephalitis. It lay on its side unable to rise, had clonic convulsions, and salivated. After a day of this it began to improve and could rise again although its hind quarters were weak and its gait unsteady. Recovery was finally complete. The course of the disease had been entirely afebrile. The other swine exhibited no evidence of active central nervous system involvement. It lay listlessly in its pen, did not eat, and was febrile for 6 days. Its recovery was uneventful and complete. From the clinical picture alone it would have been impossible to state definitely that the disease which developed in either hog was the result of infection with the "mad itch" virus. To obtain information on this point, blood serum from both animals after they had completely recovered was tested for virucidal properties, using as the control serum a sample of blood serum obtained from one of the swine prior to inoculation with the "mad itch" virus.

The results of this virus neutralization are given in Table III.

The virus-serum mixtures recorded in Table III were allowed to stand 2 hours at room temperature and 20 hours at refrigerator temperature before inoculation into animals. Consideration of the data indicates that, in the dilutions used, both samples of convalescent swine serum completely neutralized the "mad itch" virus whereas the serum from one of the animals obtained before its inoculation with "mad itch" virus was devoid of virus-neutralizing properties. It therefore seems clear that the disease which developed in the swine was actually due to the injected "mad itch" virus and that swine are susceptible to the virus although more resistant than other species tested. Such a fact suggests the possible existence of a swine disease due to "mad itch" virus. It seems probable that a potent virucidal serum can be got from swine.

No definite information concerning the susceptibility of man to infection with the "mad itch" virus has been obtained during the investigation. The writer and his helper have used ordinary precautions in handling infectious material but as autopsies have occasionally been done without gloves the bare but relatively intact skin of the hands has come in intimate contact with material of known infectivity without any untoward result. The violence of the disease, its uniformly fatal outcome, and the wide variety of species susceptible to it provide sufficient reasons for caution during work with it.

TABLE III

Neutralization Experiments with Convalescent Swine Sera

Virus – 10% suspension brain Rabbit 148	Serum	Guinea Pig No.*	Result
cc.			
1	0	249	Died—65½ hrs.
1	1 cc. normal serum Swine 810	248	" —70 "
1	1 " " " " 810	238	" —78 "
1	1 cc. convalescent serum Swine 810**	239	No symptoms
1	1 " " " " 810**	246	" "
1	1 " " " " 772***	237	" "
1	1 " " " " 772***	247	" "

* All animals inoculated subcutaneously.

** Serum drawn 17 days after inoculation.

*** Serum drawn 28 days after inoculation.

Contagiousness of Experimental "Mad Itch"

In a test of the contagiousness of experimental "mad itch" in rabbits, a doe and her litter of four were used, representing as intimate a form of contact as was attainable under experimental conditions. All were in the same cage. The young were 22 days old at the time of their mother's infection and not completely weaned although they ate freely of the rations furnished the mother. The doe received 0.2 cc. of a 10 per cent suspension of rabbit brain virus subcutaneously in the tip of one ear and after an incubation period of 58 hours developed typical "mad itch." Pruritus and self-mutilation were limited to the inoculated ear and the side of the head. The animal died after an illness of 8 hours. No disease developed in any of the young rabbits during a period of observation of 2 weeks following their mother's death. They were all then tested for susceptibility by inoculation subcutaneously and all succumbed to the "mad itch" virus. Three other experiments have been conducted in which normal rabbits have been kept in cages with intranasally infected rabbits and in no instance has disease developed as a result of contact.

No attempt has been made as yet to determine how the disease is contracted in the field. It may be of significance that on the farm where the outbreak of "mad itch" was seen in the dairy cattle there had been a highly fatal epizootic of some sort among the rat population the preceding week. However, no rat carcasses were available for examination and no live rats could be found on the place at the time that the cattle were sick.

Viability of Virus when Glycerinated or Dried

Infectious rabbit brain stored in 50 per cent glycerol at refrigerator temperature still contains active and only slightly attenuated virus after 154 days. Similar infectious material frozen and dried by Swift's method (2) still contains active virus after 106 days' storage. Dried or glycerinated material has not been tested after longer periods of storage.

The Relationship of "Mad Itch" and Pseudorabies

The clinical picture of "mad itch" is very suggestive of pseudorabies, first described by Aujeszky (3). In fact, only after some experimentation did any doubt arise that we were dealing with the latter. White (4) in a text-book of veterinary medicine has classed "mad itch" as synonymous with pseudorabies but on no experimental basis. By veterinary practitioners it is frequently considered a form of bovine hemorrhagic septicemia and, as such, treated unsuccessfully.

Professor Alădar Aujeszky of the Ungarische Tierärztliche Hochschule, Budapest, has kindly furnished me with a Hungarian strain of pseudorabies virus for the purpose of comparing the disease with "mad itch."

Pruritus localized to the region of the site of inoculation is the cardinal symptom after the subcutaneous inoculation of rabbits with either the "mad itch" or the pseudorabies virus. The incubation period and the period of survival following the onset of symptoms are the same with both viruses. Animals inoculated with pseudorabies virus exhibit more symptoms referable to central nervous system involvement and have a more "rabid" appearance than animals infected with "mad itch." Otherwise there are no observable differences in the clinical pictures. The findings at autopsy in an animal dead of "mad itch" are identical with those due to pseudorabies.

The distribution of virus in the body of an animal dead of "mad itch" differs widely from that in pseudorabies. Aujeszky (3), Schmiedhoffer (5), Zwick and Zeller (6), Sangiorgi (7), Carini and Maciel (8), and Isabolinsky and Patzewitsch (9) agree that the pseudorabies virus can be demonstrated not only in the local lesion but also in the brain, blood, and all highly vascular organs; it is a septicemic virus. The observation has been confirmed in this laboratory. In "mad itch," however, the virus is relatively localized, as indicated earlier in the paper.

Data concerning the filtrability of the etiological agent of pseudorabies are contradictory, and consideration of the literature would indicate that the virus is filtrable only with difficulty and irregularity at the best. Schmiedhoffer (5) found that the etiological agent was capable of passage through coarse pored filters which, however, retained *B. coli*, rotlauf, and fowl cholera bacilli. Sangiorgi (10), using a Brazilian strain of the disease, was irregularly successful in passing the virus through Berkefeld filters N and V. Aujeszky (3), Zwick and Zeller (6), Isabolinsky and Patzewitsch (9), and Bertarelli and Melli (11) were unsuccessful in filtering it. In a single filtration experiment conducted in this laboratory with pseudorabies, the virus has been found capable of passage through a Berkefeld filter of N porosity.

For this filtration brain tissue from an intracerebrally inoculated rabbit was suspended in infusion broth of pH 7.3 and frozen and thawed twice before filtration. All of the recorded attempts to filter the pseudorabies virus were carried out prior to 1914, that is to say prior to the recent advances in methods and technic in filtration.

Among the laboratory animals, rabbits are stated to be more susceptible to pseudorabies than guinea pigs, and guinea pigs in turn are more susceptible than white rats and mice (3, 5, 6). This same order of degree of susceptibility is true of "mad itch." However, Zwick and Zeller (6) found that rats were refractory to intraperitoneal inoculation with the pseudorabies virus, whereas in my experience the intraperitoneal injection of the "mad itch" virus almost always results in a fatal infection. Also chickens are considered insusceptible to infection with pseudorabies (3, 5, 6), but they succumb quite regularly to intracerebral inoculation with "mad itch" virus. Swine are stated (5) to be insusceptible to pseudorabies. They develop a non-fatal disease following inoculation with "mad itch" virus. Von Rátz (12), however, has observed pseudorabies in wild swine.

Aujeszky (3), Zwick and Zeller (6), and Schmiedhoffer (5) agree that pseudorabies is not contagious. "Mad itch" has proved not contagious even on intimate exposure, as the present paper shows.

Experimental infection with "mad itch" and pseudorabies can be induced by the same routes. However, Schmiedhoffer (5) has stated that intranasal inoculation of the pseudorabies virus proved innocuous unless the nasal mucous membrane was injured. The "mad itch" virus, on the other hand, regularly induces fatal infections when placed upon the intact mucous membrane of the nose. Using Aujeszky's strain of pseudorabies we have been successful in infecting a rabbit intranasally in the absence of any injury to the nasal mucosa.

It has been possible to obtain virucidal sera from both guinea pig and swine by the injection of the "mad itch" virus, and these sera are capable of completely neutralizing virulent "mad itch" virus as shown by the inoculation of mixtures of virus and serum into guinea pigs or rabbits. The virucidal swine serum is true convalescent serum while that from the guinea pig has been obtained without inducing any observable illness in the source animal. With a view to determining the relationship of the "mad itch" and pseudorabies viruses cross-neutralization tests were conducted with these sera. It was found that the sera virucidal for "mad itch" were capable of completely neutralizing the pseudorabies virus. The results of a typical experiment are given in Table IV.

There is no record in the literature of the preparation of a pseudorabies virucidal serum, and we have been unsuccessful up to now in immunizing animals.

TABLE IV

*Neutralization of Pseudorabies Virus by Anti-"Mad Itch" Sera**

Pseudorabies virus, 10% suspension of brain of Rabbit 168	Serum	Guinea Pig No.**	Result
cc.			
1	0.5 cc. normal serum Guinea Pigs 233 and 238	270	Died—81 hrs.
1	0.5 cc. anti-"mad itch" serum Guinea Pig 168	272	No illness
1	0.1 cc. anti-"mad itch" serum Guinea Pig 168	271	" "
1	1 cc. normal serum Swine 810	273	Died—93 hrs.
1	1 cc. "mad itch" convalescent serum Swine 772	274	No illness
1	1 cc. "mad itch" convalescent serum Swine 810	275	" "

* The virus-serum mixtures were allowed to stand 2 hours at room temperature and 14 hours at refrigerator temperature prior to inoculation into animals.

** All animals inoculated subcutaneously.

From the data presented in this paper the writer is led to the tentative conclusion that the virus of "mad itch" and that of pseudorabies are the same; the differences observed may be accounted for on the basis of slight differences in strains of the same virus. Further information as to their relationship must await completion of cross-neutralization tests with "mad itch" virus and pseudorabies virucidal serum.

SUMMARY AND CONCLUSIONS

The clinical picture and gross pathology of spontaneous and experimental "mad itch" have been described and the inciting agent has been shown to be a filtrable virus. It has been possible to prepare virucidal serum capable of neutralizing the virus. Fatal infections are regularly produced in rabbits when the virus is administered subcutaneously, intracerebrally, intravenously, intratesticularly, intraperitoneally, intranasally, or when it is dropped on a scarified area of skin. Its infectivity for other species by various routes is reported upon. The rabbit, guinea pig, white rat, white mouse, gray field mouse, cow, cat, duck, chicken, and hog are susceptible to experimental infection. The disease is not contagious under laboratory conditions and the virus is restricted in the animal body largely to the region of inoculation and the lung. The virus can be stored for relatively long periods in 50 per cent glycerol or in the dried state.

A comparison of "mad itch" with pseudorabies leads to the tentative conclusion that the inciting agents of both are the same, although the strains of the two viruses that are under study possess readily demonstrable differences.

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SWINE INFLUENZA

I. EXPERIMENTAL TRANSMISSION AND PATHOLOGY

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PLATES 32 TO 34

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Swine influenza ("hog flu") was first recognized as a clinical entity in the fall of 1918. Because of the prevalence at the same time of human influenza and a marked resemblance in the symptoms of the two diseases Koen became convinced that they were the same. He therefore gave the name of "flu" to the new malady of hogs (1).

The cardinal features of swine influenza are amply presented in the veterinary literature (1–6).

Swine influenza is essentially a disease of autumn and early winter and reaches epizootic proportions each year. The onset is sudden and the incidence in an affected herd is practically 100 per cent. Fever, anorexia, prostration of an extreme type, cough, and a peculiar abdominal type of respiration are salient features of the disease. The animals cry out when handled, which has been interpreted as evidence of muscular tenderness. The period of illness is short, varying from 2 to 6 days, and in uncomplicated cases the recovery is almost as sudden as the onset. The mortality is stated to range from 1 to 4 per cent. Fatal cases exhibit an extremely edematous type of bronchopneumonia.

During the autumn of 1928 and of 1929 two epizootics were observed by the writer in eastern Iowa.[1] That in 1928 was very severe, and while the mortality as a whole probably did not exceed 4 per cent, in some herds the losses were more than 10 per cent. In 1929 the epizootic was extremely mild and the mortality was less than 1 per cent.

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[1] For material and advice about the disease we are grateful to the following veterinarians of eastern Iowa: Drs. Fred J. Crow, J. S. Potter, and E. O. Thomas of Iowa City; A. H. Legenhausen, G. B. Munger, and J. W. Griffith of Cedar Rapids; H. J. Fry of Kalona, G. Lames of Dysart, J. B. Bryant of Mt. Vernon, R. Schuchert of Keystone, J. C. Glenn of Norway, and R. E. Elson of Vinton; also to Mr. N. W. Brooks of Cedar Rapids.

We are indebted to the State University of Iowa Medical School, and especially to Dr. O. H. Plant of the Department of Pharmacology and Dr. G. H. Hansmann of the Department of Pathology, for laboratory facilities during the autumns of 1928 and 1929.

The term "hog flu," as popularly used, embraces more than one clinical entity. This point was particularly emphasized by our 1929 field observations. While in 1928 the epizootic disease was so plentiful in eastern Iowa that material from typical cases was easily obtained, in 1929 much difficulty was encountered. Many cases were then seen in which a loose diagnosis of "hog flu" had been made. These animals, as a rule, were found to be suffering from a respiratory affection, quite often simulating the true epizootic swine influenza in some respects but differing markedly in others. The true nature of the condition could usually be recognized by the absence of prostration on the part of the affected animals, the greater chronicity of the disease, and the failure of more than a small portion of the herd to become affected.

Experimental Transmission

Swine influenza has been established without difficulty by experimental infection of swine with eight separate strains of the disease brought from Iowa.

Infectious material was transported in tubes packed in iced thermos jugs and was usually *en route* for from 36 to 48 hours. The method used in inducing infections was that described by McBryde, Niles, and Moskey (6). It consisted in the intranasal instillation of either suspensions of bronchial mucus alone or mixtures of suspensions of bronchial mucus, bronchial lymph nodes, and diseased lung. The instillations were made with a Luer syringe without needle by pressing the tip of the syringe into the external nares. The suspensions were prepared in either distilled water or infusion broth and were made up to between 10 and 20 per cent. The dosage of suspension administered usually ranged from 5 to 30 cc., but typical and severe influenza was produced by the intranasal injection of doses as small as 1 cc. Only a small portion of the material administered was retained, for most of it was either sneezed or blown out of the nostrils.

After the establishment of the disease animals from which material for further inoculations was to be taken were slaughtered on the 3rd or 4th day following the first appearance of a temperature in excess of 40°C. The disease has been maintained for study by this type of serial passage.

Because of the highly contagious nature of the disease it has been necessary to exercise extreme precaution in the isolation of individual experimental animals. Our individual isolation units have been used. We have followed the technique of isolation used here for the study of experimental hog cholera. This practice has been tested repeatedly for both hog cholera and swine influenza and found sufficient to insure complete isolation.

Unlike McBryde and his coworkers (6) we have encountered no spontaneously immune animals during the investigation. The probable explanation is that McBryde investigated the disease during the epizootic period in Iowa and used as his experimental animals swine from the same region. These animals may have been in contact with the disease prior to their use experimentally and may in consequence have developed some degree of immunity. Such a possibility was eliminated in our own experiments. All of the transmission work was conducted at Princeton, New Jersey, and the experimental animals were either raised on the Institute farm or purchased from nearby farmers. Epizootic swine influenza does not occur in the locality.

The method of inoculation has been adequately controlled. Normal swine have been injected intranasally with large doses of suspensions of lung and bronchial lymph nodes from normal swine and in no instance has any evidence of illness developed or anything suggestive of influenzal disease been encountered at autopsy.

Clinical Features of Experimental Swine Influenza

The symptoms induced by the several strains of swine influenza have varied in degree but very little in type.

Strain 1 (1928) induced a more severe and virulent swine influenza than Strain 2 (1928) and Strain 2 in turn was more virulent than Strains 5, 6, 7, and 10 (1929). Strains 14 and 15 (1930) induced a disease which was of about the same severity as that induced by Strain 1. The actual mortality of the experimental disease was not determined, for most of the experimental swine were killed on the 2nd to 4th day of fever for bacteriological and pathological studies. However, fatal infections resulted five times following infection with Strain 1, four times following infection with Strain 14, and six times following infection with Strain 15. The mortality can be safely estimated as higher than that in the naturally occurring field disease.

The incubation period was found to be short. For animals infected by pen contact it varied from 2 to 7 days with the average at 4 days. Animals infected by intranasal instillation became ill more promptly and usually within 24 to 48 hours exhibited a sharp rise in temperature.

Fever, *i.e.*, a temperature of 40°C. or higher, was in all cases the first observable evidence of illness. Accompanying the rise in temperature, or following it very shortly, there was a mild degree of malaise, mild anorexia, and a tendency for the animal to tire easily when made to exert itself. While there was considerable variation in the temperature reaction, as a rule on the 1st day it reached 40.4°C. but seldom exceeded 41°C. On the 2nd day of illness the fever, anorexia, and malaise were more marked. By the 3rd day the temperature had as a rule reached its peak and frequently exceeded 41.5°C. and sometimes even 42°C. At this time respiratory involvement was manifested by increased rate and a peculiar type of diaphragmatic breathing, described popularly as "thumping," and frequently a paroxysmal type of cough was elicited when the animals were roused. They exhibited marked prostration, refused food, and lay listlessly in their pens. Their condition on the 4th and 5th days was little altered from that on the 3rd day. Death occurred on the 3rd, 4th, 5th, or 6th days and was preceded by an exaggeration of all respiratory symptoms, an increase in the prostration, and the onset of an active, incoordinated delirium during which the animal lay on its side and made running motions with its legs or, attempting to stand, would stagger about the isolation unit. On the 6th day as a rule, the temperature was definitely receding in animals that were to recover and from then on recovery was rapid.

TABLE I

Leucocytic Reaction in Experimental Swine Influenza

Swine No.	Before inoculation	Days after inoculation				
		2	3	4	5	6
	Leucocytes per c.mm.	Leucocytes per c.mm.	Leucocytes per c.mm.	Leucocytes per c.mm.	Leucocytes per c.mm.	Leucocytes per c.mm.
459	28,000	24,200	22,000	13,300		
461	19,000	14,000	10,800	12,040		
549	24,400		13,400	14,600	7,400	
555	23,000	17,900	17,300	19,800		21,400
562	28,800	27,600	20,300		11,400	
572'	22,600	15,540		11,840		14,200
583	21,920		17,600		15,500	
587	14,940		9,240		13,400	

In both the natural and the experimental disease a mild leucopenia was usually observed. A record of a few counts on experimental cases is given in Table I. There was no significant difference in the degree of leucopenia of the various cellular types as shown by differential counts.

Pathology

Descriptions of the lesions of swine influenza are fragmentary and deal entirely with fatal cases of the disease.

Dreher (5) has reported the main features as congestion of the mucous membranes of the respiratory tract, a lobular type of pneumonia, swollen and edematous bronchial lymph nodes, and congestion of the gastric mucosa. Dimock and Healy (3) have described the lungs as congested, edematous, fully distended and heavy, and frequently exhibiting bronchopneumonia. Quinn (1) has emphasized the occurrence of extensive edema of the lungs and Murray and Biester (7) found edema to be frequent. The latter authors also called attention to the similarity existing between the "water logged" lung of the human influenzal pneumonia of 1918 and that of the pneumonia of swine influenza.

It has seemed important to study the lesions at various stages of the illness. The large majority of the animals sacrificed would have progressed to an uneventful recovery. The findings in fatal cases will be described separately.

Gross Pathology.—The swine were sacrificed on the 1st to the 5th day of fever. The large majority were chloroformed while a few were stunned by a blow on the head and then bled to death. There were no external signs of disease at the time of slaughter save in some of the animals which seemed most ill. These sometimes exhibited a watery mucous nasal discharge.

The mucosa of the pharynx and larynx was, as a rule, very mildly hyperemic and covered by a white, glassy, tenacious mucus. On opening the trachea the same type of tenacious mucus was present in from moderate to copious amounts. It was sometimes frothy, very rarely blood-tinged. There was more exudate present in the large bronchi and, in the smaller bronchi and bronchioles, it completely filled the lumen. In the bronchioles the exudate was of firmer consistency than higher in the respiratory tract. It not infrequently could be removed in small, white, semitranslucent, sago-like masses. The pleural sacs were free of excess fluid or fibrin. The lungs presented very constant and characteristic gross changes. The involved lung tissue was a deep purplish red in color, noticeably depressed when compared with the uninvolved lung tissue, and the line of demarcation between normal and pathological lung substance was very definite. Palpation of the involved lung revealed that it felt "leathery," did not crepitate, and contained no areas of consolidation. The elements of the bronchial tree could be palpated and gave the impression of being thickened. On cut section the bronchioles protruded from the surface and the lung substance itself had a purplish red, "beefy," pasty appearance throughout. The gross picture was that of a marked massive atelectasis, irregular both as to amount and distribution. It was usually limited to portions of the cephalic, cardiac, and azygos lobes, and it not infrequently involved all five of these lobes. In over half of the cases it was bilateral and irregularly symmetrical. If, however, it tended to be unilateral, the right side was almost always predominantly involved. There were a number of cases in which only the right cephalic and the cardiac lobe exhibited massive atelectasis. An illustration of a typical case is presented in Figs. 1 and 2.

The adjoining non-atelectatic portions of the lungs were emphysematous. The surfaces of these portions of the lung were elevated and extremely pale. A feature occasionally observed was a moderate to extreme petechiation of the pleurae overlying the areas of compensatory emphysema. In some cases a moderate interlobular edema of the non-atelectatic areas of lung was encountered.

The cervical, mediastinal, and mesenteric lymph nodes were extremely enlarged and very edematous. They were only rarely congested. Those at the hilum of the lung were sometimes so large as to resemble grapes. On section they were found to be soft and to ooze fluid in relatively large amount.

A small excess of fluid was not infrequently encountered in the peritoneum. This was as a rule clear and straw-colored but sometimes slightly cloudy and contained flecks and strands of fibrin. There was usually, but not invariably, a moderate acute splenic tumor. The kidneys and liver were negative in the gross. The stomach was sometimes found to contain a bile-tinged watery mucus and the gastric mucosa was almost invariably extremely hyperemic especially along the greater curvature and at the cardiac end. The small intestine was negative.

The mucosa of the colon frequently exhibited hyperemic patches of various sizes. These areas were mildly edematous and overlaid by a scant catarrhal exudate. They never showed a true fibrinous membrane nor were they eroded. The mucosa was elevated over localized areas of submucous lymphoid hyperplasia.

Histopathology.—

Films of bronchial exudate stained for 24 hours in methylene blue revealed a rather constant and characteristic picture. The predominant cell in the exudate was the polymorphonuclear leucocyte and of these there were many. They were as a rule well preserved and not infrequently contained engulfed organisms, usually small thin bacilli but occasionally larger bacillary forms or cocci. There were moderate numbers of lymphocytes in the exudate and smaller numbers of desquamated epithelial cells. Lying between the cells of the exudate were large numbers of extremely thin, very faintly staining, hair-like structures, evidently broken off cilia. In some preparations these were extremely numerous. Organisms of the type seen in the leucocytes were sometimes very numerous between the cells of the exudate but as a rule they were scarce.

Tracheal sections showed little that appeared abnormal.

Lung sections cut in such a way as to include small bronchi and terminal bronchioles, and including uninvolved and typically diseased lung exhibited the following features. The small bronchi and terminal bronchioles were filled with a polymorphonuclear leucocytic exudate (Figs. 7 and 8). Bacteria were never numerous in this exudate and frequently they were not demonstrable, or were present in such small numbers as to require careful search to find them. They were most numerous at the junction of the exudate and the bronchial epithelium. The cilia lining the smaller bronchi were either entirely gone or badly matted together. The lining epithelium was fragmented, in places partially desquamated, and the cytoplasm of many of the cells appeared vacuolated (Figs. 4 and 5). In the spaces created by the fragmentation of the lining epithelium, leucocytes, singly or in clumps, were sometimes seen (Fig. 5). There was an extensive peribronchial round cell infiltration (Figs. 4, 5, 7, 8). The areas of lung that appeared to be merely atelectatic were found histologically to present other changes than atelectasis alone (Figs. 9 and 10). They were of lobular distribution, and sharply demarcated from adjacent uninvolved lung by interlobular septa, although a number of adjacent lobules might be, and usually were, involved. In these areas the alveoli were collapsed and frequently contained desquamated epithelial cells, small numbers of mononuclear wandering cells, and occasionally some coagulated plasma. Large, feebly stained cells exhibiting a "foamy" cytoplasm were especially numerous in some sections. Leucocytes and red cells were not found regularly in the alveoli although it was difficult to find sections, even from very early cases, in which the alveoli in some areas of the section did not contain leucocytes and occasionally red cells in small numbers. Leucocytes when present were most abundant in the alveoli opening directly into the terminal bronchioles. The alveolar walls were wrinkled and broadened and definitely infiltrated with mononuclear cells (Fig. 6). This infiltration was most marked in the alveolar walls adjacent to the bronchi but it was present and frequently conspicuous throughout the entire area of atelectasis. Even in non-atelectatic areas of lung there sometimes was some slight to moderate distension of the alveolar walls due to a round cell infiltration. There was a moderate passive congestion of the atelectatic areas as evidenced by dilated and injected pulmonary capillaries. The lymph channels, especially in the interlobular septa, were sometimes dilated and filled with lymph and small numbers of cells. They were not observed to be thrombosed or to contain bacteria. The interlobular septa were frequently widened owing not only to the dilation of the lymph channels but to an apparent pulling apart of the connective tissue elements and to some round cell infiltration. The pleura overlying the atelectatic areas was sometimes wrinkled and thrown into small folds (Fig. 10). It was apparently unaltered otherwise. Lobules lying adjacent to areas of atelectasis were markedly emphysematous, exhibiting extremely thin alveolar walls, many of them broken.

It should perhaps be indicated here that even when all five of the upper lobes of the lung of swine are involved, over half of the actual lung substance remains relatively intact, for the diaphragmatic, or lower lobes, are greater in volume than the five upper lobes. Since it is unusual to have complete atelectasis of all five of the upper lobes, in most cases no more than one-third of the total lung volume is actually involved.

Outside the respiratory tract the histopathology was in accordance with the gross pathological picture. The cervical, mediastinal, and mesenteric lymph nodes were as a rule packed with lymphocytes, the germinal follicles appeared active, and there was much intercellular edema. The histological picture presented by the spleen was that of a slight to moderate, acute splenic tumor characterized by a moderate increase in the mononuclear cells in the pulp and very little, if any, alteration in the Malpighian bodies. The liver was sometimes passively congested but otherwise negative. The kidneys were negative. The mucosa and submucosa of both the stomach and colon showed an increase in mononuclear round cells which was sometimes quite extreme. The blood vessels in these regions were usually dilated and packed with red cells. The goblet cells in the mucosa of the colon were prominent and distended.

As this description shows, the pulmonary pathology differs markedly from that encountered at autopsy in animals succumbing to either the experimental or spontaneous disease.

The Pathology of Fatal Cases.—Death in fatal cases was not necessarily due to pathological changes induced by secondary bacterial invaders, for swine influenza of itself can kill.

The postmortem picture presented by swine dead of the experimentally induced disease was the same as in fatal spontaneous cases. The cervical lymph nodes were much enlarged, edematous, and frequently congested. The mucosa of the trachea and larger bronchi was moderately congested and covered with a thick, tenacious, and sometimes frothy mucous exudate. The smaller bronchi contained a more fluid, blood-tinged exudate and this sometimes in copious amounts. A serosanguineous pleural exudate was frequently encountered. This exudate sometimes contained considerable fibrin and in these cases the anterior lobes of the lung were covered with closely adherent fibrin. Both pleurae were usually involved, although sometimes the process was unilateral. The lungs themselves were voluminous, heavy, and mottled purplish red in color. Palpation revealed that only the apical, azygos, or cardiac lobes were consolidated. That is, the true pneumonia was limited entirely to the portions of lung which in uncomplicated swine influenza would have been atelectatic. The diaphragmatic lobes, which in swine comprise well over half the actual lung substance, exhibited a hemorrhagic type of pulmonary edema which was in most instances extreme. The markings of the interlobular septa were widened by fluid and the lobes as a whole had a glistening swollen appearance. When they were cut across there was an outpouring of a frothy, bloody fluid. There was, as a rule, no fibrin adherent to the diaphragmatic lobes.

Histological examination of sections of the pneumonic areas revealed the pleurae to be usually overlaid with a rich network of fibrin in the meshes of which were myriads of leucocytes. The bronchioles were completely filled with leucocytes, the lining epithelium was badly fragmented and partially desquamated, and the bronchial walls were densely infiltrated with round cells. The alveoli throughout the section were filled with leucocytes, red blood cells, and coagulated plasma. There was usually no fibrin in the alveolar exudate. The alveolar walls were mildly folded, thickened, and infiltrated, largely with round cells. The lymph sinuses in the interlobular septa were dilated and contained leucocytes, some lymphocytes, and much lymph.

Histological examination of sections of the edematous portions revealed plasma filling the alveoli which also contained small numbers of desquamated epithelial and red cells. There was no fibrin. The cellular exudate in the bronchi was scant but there was much plasma. The bronchial walls were thickened owing to intercellular edema and some round cell infiltration. The pulmonary capillaries were usually dilated and packed with red cells. The lymph sinuses in the interlobular septa and beneath the pleura were widely dilated and contained, in addition to plasma, small accumulations of leucocytes and lymphocytes. The lymph nodes at the hilum of the lung were always enlarged and edematous and usually moderately congested. In some cases, especially those in which a fibrinous pleuritis had been encountered, a serofibrinous pericarditis was also to be observed. The livers of fatal cases of swine influenza were usually engorged and the spleens large, swollen, dark, and friable. An acute diffuse nephritis was occasionally encountered. The stomachs were usually empty except for the presence of moderate to copious amounts of a thin bile-tinged mucus. The small intestines also usually contained considerable bile-tinged mucus. The mucosa of the cecum and the first two-thirds of the colon was intensely hyperemic.

Animals slaughtered during convalescence have much interest from a pathological standpoint. Pulmonary evidence of the disease persisted for 3 or 4 weeks or longer following clinical recovery. Areas of lung were found that were slightly depressed as compared with the adjoining normal tissue. They were grayish pink, on palpation felt firmer than normal, and cut as though fibrous. The bronchi in some instances were dilated and contained a mucopurulent material. Sections revealed much fibrous tissue and great thickening of the alveolar walls.

In animals convalescent from a swine influenzal pneumonia, lung abscess, bronchiectasis, or an obliterative pleuritis were not infrequently encountered at autopsy.

SUMMARY

Swine influenza has been induced in pigs by the intranasal instillation of material from spontaneous cases of the disease as occurring epizootically in eastern Iowa. The experimental disease has the same features as the epizootic. It has been maintained for study by serial passages accomplished either by intranasal instillation or by pen contact. Eight strains of the virus have been established experimentally during three epizootic periods. The clinical disease induced by these eight strains has been in general the same although its severity and mortality have varied.

The principal features of the pathology of swine influenza are an exudative bronchitis accompanied by marked damage of the bronchial epithelium and its cilia, a peribronchial round cell infiltration, and massive pulmonary atelectasis. The latter is modified somewhat by a round cell infiltration of the alveolar walls. The lymph nodes, especially the cervical and mediastinal ones, are hyperplastic and edematous. There is usually a mild to moderate, acute splenic tumor. The mucosa of the stomach and colon is congested.

The pneumonia following swine influenza is, characteristically, lobular in type and of the same general distribution as the atelectasis. The non-pneumonic areas of lung are extremely edematous and congested.

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EXPLANATION OF PLATES

PLATE 32

FIG. 1. Dorsal aspect of lung in experimental swine influenza (Swine 895) to show the typical appearance and distribution of the massive pulmonary atelectasis. The lymph nodes at the hilum are swollen and edematous. The sharp demarcation between atelectatic and non-atelectatic lung is noteworthy. Animal chloroformed on the 4th day of illness.

FIG. 2. Ventral aspect of same lung.

PLATE 33

FIG. 3. Section of a small bronchus from a normal hog (Swine 588) showing intact ciliated epithelium overlying a normal submucosa. Animal killed by stunning and bleeding. Eosin-methylene blue. × 305.

FIG. 4. Section of a small bronchus from a spontaneous field case of swine influenza (Swine 13) showing fragmented epithelium and extreme round cell infiltration of the submucosa. Animal stunned and bled. Eosin-methylene blue. × 305.

FIG. 5. Section of a small bronchus in experimental swine influenza (Swine 564) showing leucocytic bronchial exudate, fragmented and vacuolated epithelium denuded of cilia, and round cell infiltration of the submucosa. Leucocytes have invaded the mucosa. Animal chloroformed on 5th day following inoculation. Eosin-methylene blue. × 305.

FIG. 6. Section of lung in experimental swine influenza (Swine 473) showing thickening of the alveolar walls in an area of atelectasis. The cells infiltrating the alveolar walls are largely round cells. Animal chloroformed on 3rd day following inoculation. Eosin-methylene blue. × 295.

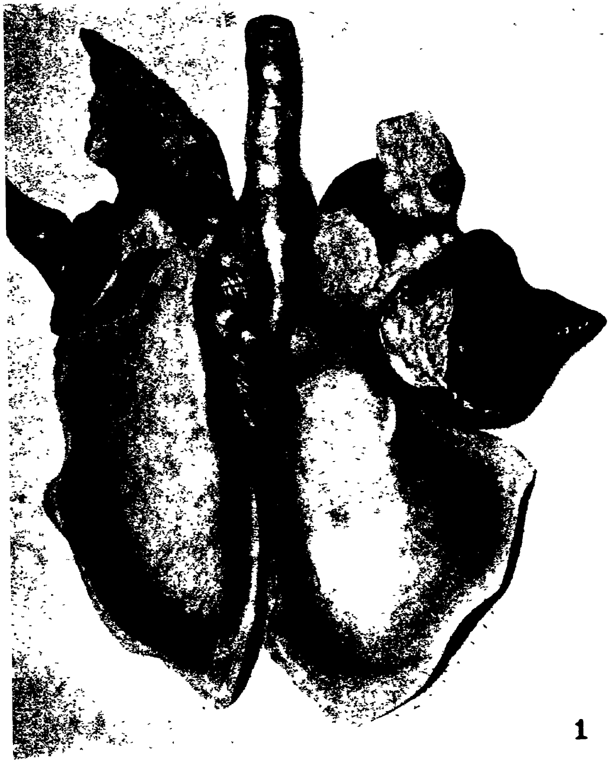
PLATE 34

FIG. 7. Section of lung from a spontaneous field case of swine influenza (Swine 13) showing a bronchus in an area of compensatory emphysema to illustrate better the dense peribronchial round cell infiltration. The lumen of the bronchus contains a dense leucocytic exudate. Animal stunned and bled. Eosin-methylene blue. × 51.

FIG. 8. Section from the lung in experimental swine influenza (Swine 593) showing dense leucocytic exudate in small bronchus and peribronchial infiltration largely with round cells. Animal chloroformed on 6th day following inoculation. Eosin-methylene blue. × 51.

FIG. 9. Section of lung from a spontaneous field case of swine influenza (Swine 97) showing atelectasis with infiltration of the alveolar walls, slight leucocytic exudate in some of the collapsed alveoli, and compensatory emphysema. Animal stunned and bled. Eosin-methylene blue. × 51.

FIG. 10. Section of lung in experimental swine influenza (Swine 454) showing folding of pleura over an area of atelectasis. Animal chloroformed on 3rd day following inoculation. Eosin-methylene blue. × 51.



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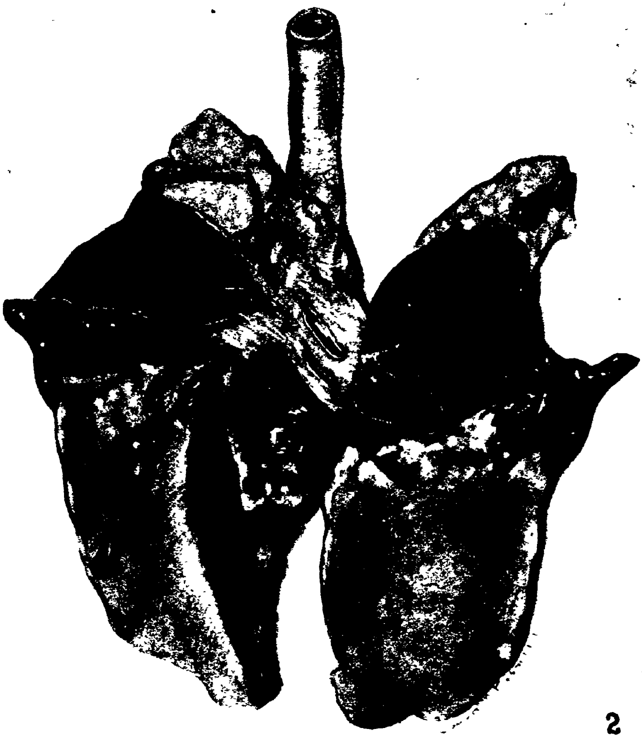

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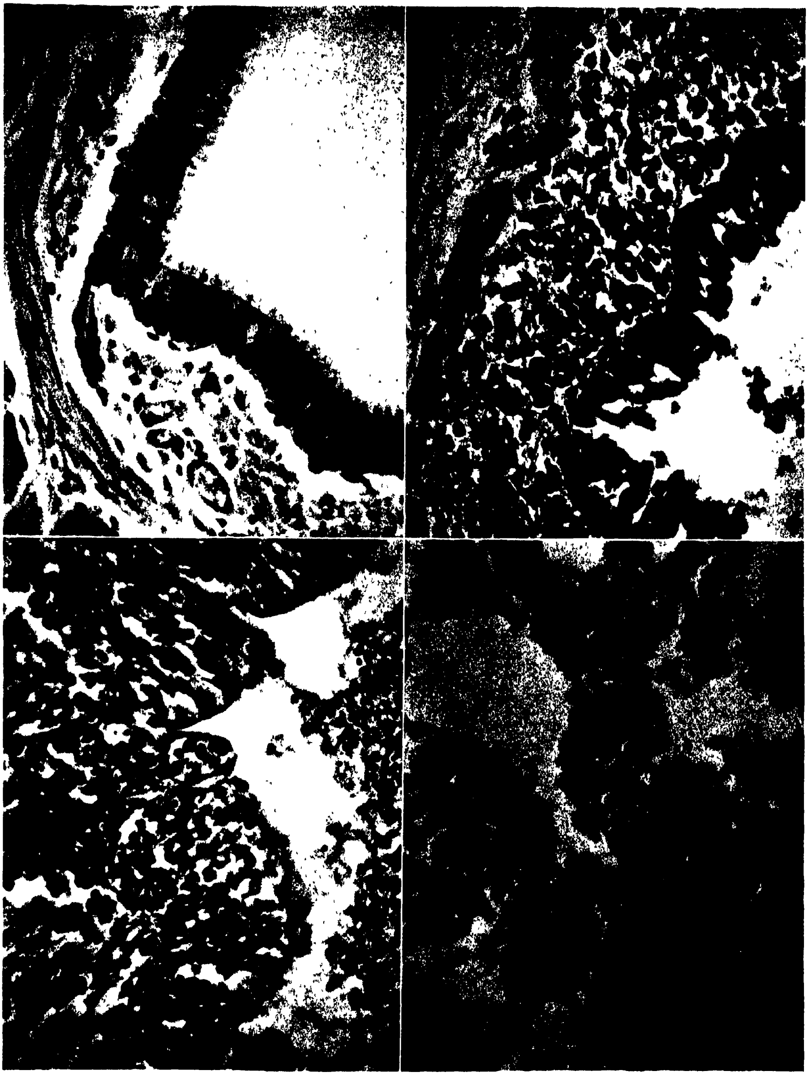
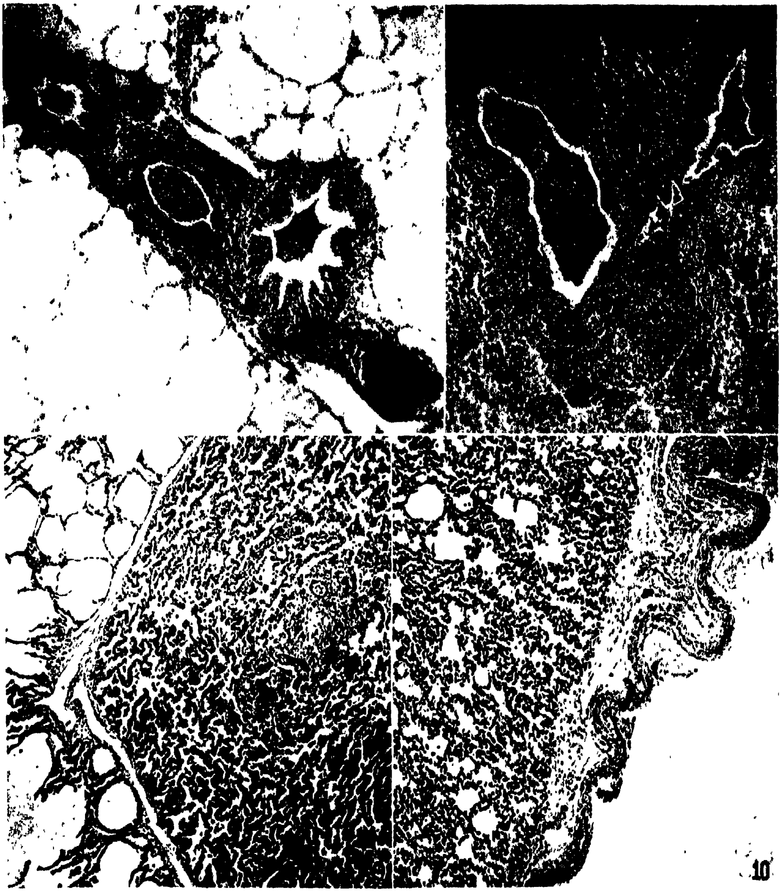


(Shope: Swine influenza. I)



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SWINE INFLUENZA

II. A Hemophilic Bacillus from the Respiratory Tract of Infected Swine

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Plate 35

(Received for publication, May 6, 1931)

Murray (1), McBryde, Niles, and Moskey (2), and recently Fulton (3) have attempted to discover the nature of the inciting agent of swine influenza. Murray described a small Gram-negative coccus as the cause of the disease. By inoculating normal swine intravenously with relatively small doses of pure cultures of this organism, he induced a disease which he believed was clinically and pathologically swine influenza. McBryde, Niles, and Moskey failed to obtain Murray's micrococcus in their cultures. They isolated only two types of organisms from cases of the disease with any degree of frequency, a pleomorphic Gram-positive bacillus and *Bacillus suisepticus*. Neither of these organisms produced the disease when injected into susceptible swine. Fulton recently confirmed the presence of McBryde's pleomorphic Gram-positive bacillus in the respiratory tracts of swine with influenza and, with very freshly isolated cultures, reproduced the disease experimentally. However, cultures that had been transferred only three times or original cultures that had been kept on ice for as short a period as 2 weeks were incapable of inducing influenza when administered intranasally to normal swine.

Spray (4) using slaughter house material cultured the respiratory tracts of a large number of normal swine and of those showing pneumonia. From both classes he frequently isolated an inulin-fermenting streptococcus and *B. suisepticus*. These organisms were encountered more often from pneumonic than from normal lungs; the inulin-fermenting streptococcus was frequently present in pure culture from the pneumonic areas. Since Spray did not differentiate infectious pneumonia, hemorrhagic septicemia, and influenza, his findings are not of great value.

EXPERIMENTAL

Bacteriological studies have been conducted upon a large number of swine experimentally infected with swine influenza from the 1928, 1929, and 1930 epizootics (5), and upon a small number of spontaneous field cases of the disease from the mild 1929 epizootic. In addition, thirty-five swine not suffering from influenza have served as a control group. These animals were either normal, or infected with hog cholera or spontaneous pneumonia. They were from local sources similar to those of the animals used in the study of experimental swine influenza.

In suspected cases of influenza, primary cultures were made from atelectatic or pneumonic lung, bronchial exudate, spleen, and, in certain cases where indicated, from other organs, on plain agar slants (pH 7.3 to 7.6), to the condensation fluid of which 0.5 to 1 cc. of sterile defibrinated horse or swine blood had been added. Cultures of heart blood were made on plain agar slants inoculated by pipette with approximately 1 cc. of blood. In some cases primary cultures were also made on blood and chocolate agar plates. Similar cultures were made from the control animals on the same kinds of media.

The suspected organism was very similar to, if not identical with, *H. influenzae*. The first culture was obtained from the heart blood of our first experimental case of the disease. This blood was apparently sterile when streaked on plain and blood agar plates. In approximately 1 cc. amounts it was added to the condensation fluid of about twenty plain agar slants which were incubated for 2 days. At this time the slants appeared sterile. The blood was not hemolyzed and there was no evidence of growth. However, films prepared from the bloody condensation fluid of these slants, when stained with methylene blue and examined microscopically, revealed the presence of organisms in moderate numbers. These were predominantly thin, curved bacilli which varied considerably in length. Some were less than 1μ long while other apparently single individuals sometimes approached 4μ in length. Some threads of continuous protoplasm extended completely across the field. Other shorter threads appeared as tangled masses enmeshing small curved bacillary forms and both large and small coccoidal forms. Attempts to obtain subcultures on plain agar, infused blood agar, inspissated serum, or in bouillon or Smith-Noguchi medium were unsuccessful. Growth could be continued, however, in sterile defibrinated swine or horse blood at the bases of plain agar slants and fair growth reaching a maximum in 48 hours occurred on chocolate agar.

This organism has been found in the respiratory tracts of all swine experimentally infected with influenza that have come to autopsy within 7 days of the onset of fever; it has been recovered from the respiratory tracts of six spontaneous cases of the disease from five different herds (all of the spontaneous cases which were examined); and it has been found in the original material used in establishing the eight strains of the disease which have been studied experimentally (5). In the case of all eight strains the organism has been recovered from the respiratory tract of the first experimentally infected animal

TABLE I

The Incidence of H. influenzae (Variety suis) in Experimental and Spontaneous Swine Influenza

Strain	No. of swine cultured	Material cultured	*H. influenzae suis*				
			Present	Absent	Per cent positive	Present in pure culture	Per cent present in pure culture
1 (1928)	33	Lung	28	4	87	11	34
		Bronchial exudate	17	6	74	8	35
		Heart blood	6	20	23	5	19
2 (1928)	9	Lung	7	2	78	4	44
		Bronchial exudate	8	1	89	3	33
		Heart blood	0	9	0	0	0
5, 6, 7, and 10 (1929) combined	7	Lung	7	0	100	5	71
		Bronchial exudate	6	0	100	2	33
		Heart blood	0	7	0	0	0
Field cases (1929)	6	Lung	5	1	83	1	17
		Bronchial exudate	6	0	100	2	33
		Heart blood	2	4	33	0	0
14 (1930)	8	Lung	6	2	75	5	63
		Bronchial exudate	8	0	100	2	25
		Heart blood	2	5	29	2	29
15 (1930)	25	Lung	23	2	92	15	60
		Bronchial exudate	24	0	100	9	38
		Heart blood	8	17	32	8	32

TABLE II

The Incidence of Hemophilus influenzae (Variety suis) in a Control Group of Animals

Group	Material cultured	*H. influenzae suis*		
		Present	Absent	Percentage
Swine experimentally infected with hog cholera—16	Lung	0	16	0
	Bronchial mucus	0	13	0
Normal swine—8	Lung	0	8	0
	Bronchial mucus	0	8	0
Swine dying of or showing pneumonia at autopsy—11	Lung	0	11	0
	Bronchial mucus	0	8	0
Iowa swine suffering from a non-influenzal respiratory disease—4	Lung	0	4	0
	Bronchial mucus	0	4	0

in each series. In these groups of cases it has frequently been present in pure culture. Because of its growth requirements, porcine origin, and morphological and cultural characteristics, to be discussed later, the name *Hemophilus influenzae* (variety *suis*) is suggested for this organism. It will be referred to hereafter as *H. influenzae suis.* The incidence of *H. influenzae suis* in influenza-sick swine killed within 7 days after inoculation is recorded in Table I. In addition to the cultures recorded in Table I, it has been recovered from the pleura or pericardium whenever these have contained exudate, and from the spleens of most fatal cases. In two cases which developed otitis media during the course of their experimental disease it was the predominant organism in the middle ear exudate. It has not been encountered in the swine serving as bacteriological controls, as indicated in Table II.

Cultural and Morphological Characters of H. influenzae suis

Morphologically and in the character of its growth on media *H. influenzae suis* resembles *H. influenzae* very closely.

Media to sustain growth must contain both factors V and X (6). Hematin prepared by the method of Anson and Mirsky (7) and supplied by them was used as the source of factor X, while factor V was furnished either by an actively growing culture of a non-hemolytic streptococcus (spotted on the hematin-containing plate) or by a sterile Berkefeld filtrate of an old bouillon culture of this streptococcus added to hematin-containing agar.

Chocolate agar is the best solid medium and heated blood bouillon the most satisfactory fluid medium for the growth of *H. influenzae suis.* Cultures of the organism that have been well established grow feebly and as very minute colonies on blood agar. In mixed culture on blood agar luxuriant growth of *H. influenzae suis* colonies occurs about the colonies of the contaminating organism. On chocolate agar well established cultures reach a maximum in between 24 and 48 hours at 37°C. The colonies on this medium vary from less than 1 mm. to more than 2 mm. in diameter, are circular, grayish, semitranslucent, flattened, and have a sharply contoured edge. Films prepared from chocolate agar cultures contain three distinct forms. Examined after 24 hours' growth the predominant form is a small thin bacillus varying from less than 0.5μ to 2μ in length and approximately 0.2μ in thickness (Fig. 1). A few longer, curved, thread-like forms appear also. 48 hour cultures contain more of the long thread-like types and in some cultures these form clumps of tangled masses of organisms (Fig. 2). Small coccoidal organisms are found at 48 hours. Cultures examined at 3 days and thereafter reveal increasing numbers of the coccoidal forms. In blood bouillon or heated blood bouillon 24 hour cultures are found to be composed largely of small, thin, straight or slightly curved rods. A few coccoidal forms are present and these usually in clumps of from six to thirty or more organisms, seldom singly. At 48 hours almost the entire culture is composed of clumps of small cocci (Fig. 3). In fluid media the long thread-like forms are not regularly encountered. "Giant" coccoidal and large club and comma shaped forms are numerous in some cultures (Fig. 4).

The capacity of films of cultures from either solid or fluid media to absorb the usual dyes decreases rapidly with the age of the culture. Loeffler's alkaline methylene blue, which is the most satisfactory, imparts no more than a faint bluish gray color to organisms from cultures 3 or more days old. By phenolizing the methylene blue and steaming it on the film a slightly more intense color is obtained.

The organism is relatively inert as to growth and fails to act on various substances used for differentiating bacteria. In blood bouillon, with brom-cresol purple as indicator, definite growth occurs. There is, however, no demonstrable action on dextrose, lactose, saccharose, dulcitol, mannitol, glycerol, inulin, or arabinose. Some fading of the indicator occurred in all tubes. All cultures were observed for 7 days. None of the cultures examined produced indol or hydrogen sulfid. Litmus milk containing blood fostered but slight growth and was unchanged. Nitrates were reduced to nitrites by all cultures examined. No growth could be obtained with well established cultures on plain or glycerin agar, Dorset's egg medium, potato, coagulated blood serum, or in gelatin. The organism is non-hemolytic, non-motile, and Gram-negative.

As may be evident from the preceding description, there is no real basis for differentiation between *H. influenzae suis* and non-indol-producing strains of *H. influenzae.* If a culture of *H. influenzae suis* were isolated from a human throat, no doubt would be raised as to its identity with *H. influenzae.* Since primary cultures of *H. influenzae suis* grow somewhat more feebly on heated blood agar than do primary cultures of *H. influenzae,* such a hypothetical culture would perhaps be considered more difficult to establish than the typical Pfeiffer bacillus.

For the primary isolation of *H. influenzae suis,* the plain agar slant containing from 0.5 to 1 cc. of sterile defibrinated horse blood at the base is the medium of choice. In this medium the organism grows in the blood at the base of the slant and the pure culture on inspection appears sterile. Examination of stained films of the bloody condensation fluid, however, readily reveals the organism in characteristic form. It is felt that for the isolation of *H. influenzae suis* this medium is superior to heated blood agar. It has been the practice not to examine

TABLE III

Direct Agglutination

Culture strains	Agglutinating sera																																			
	H. influenzae suis																												*H. influenzae* BIW							
	14							451							459							660														
	Dilutions																																			
	1:50	1:100	1:200	1:400	1:600	1:800	1:1200	1:50	1:100	1:200	1:400	1:600	1:800	1:1200	1:50	1:100	1:200	1:400	1:600	1:800	1:1200	1:50	1:100	1:200	1:400	1:600	1:800	1:1200	1:50	1:100	1:200	1:400	1:600	1:800	1:1200	Control
H. influenzae suis																																				
11-T	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
652	C*	C	+	+	—	—	—	C	C	C	C	+	—	—	C	C	C	C	+	—	—	C	C	C	C	+	—	—	C	C	C	C	+	—	—	—
451	—	—	—	—	—	—	—	C	C	C	C	+	x	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	C	C	+	—	—	—	—	—
459	—	—	—	—	—	—	—	+	x	—	—	—	—	—	C	C	C	+	—	—	—	—	—	—	—	—	—	—	C	+	x	—	—	—	—	—
660	C	C	C	+	—	—	—	C	C	C	C	+	—	—	C	C	C	C	+	+	—	C	C	C	C	+	x	—	+	C	C	+	—	—	—	—
11-I	x	+	—	—	—	—	—	C	C	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—	—	—	—	—
15	C	C	+	—	—	—	—	+	+	x	—	—	—	—	C	C	+	—	—	—	—	C	+	—	—	—	—	—	C	C	+	—	—	—	—	—
14	C	C	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	C	C	+	—	—	—	—	C	C	+	—	—	—	—	—
H. influenzae																																				
WF	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
K	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	x	+	—	—	—	—	—	—
S	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	C	C	C	+	—	—	—	—
14	C	C	+	—	—	—	—	C	+	+	—	—	—	—	+	—	—	—	—	—	—	+	x	—	—	—	—	—	C	C	C	C	C	C	C	—
52	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	C	C	+	—	—	—	—	—
BIW	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—	C	C	C	C	C	C	C	—

* C = complete agglutination; +, strong agglutination; x, weak to trace agglutination.

primary cultures until after 48 hours' incubation, since this length of time is required for the development of the curved and tangled thread-like forms which are characteristic and identifiable even in mixed culture. Cultures are viable in this medium for 2 weeks, but stock cultures have been transferred at weekly intervals and kept in the incubator.

Serological Relationship between Strains of H. influenzae suis and of H. influenzae

It was realized that, because of the great serological diversity of strains of *H. influenzae*, attempts to demonstrate any serological relationship between *H. influenzae suis* and *H. influenzae* would be inconclusive. However, the near identity of the two organisms both culturally and morphologically made it seem of interest to compare the reciprocal agglutinability of a few strains of each organism. The cultures of *H. influenzae* and the agglutinating serum were generously provided by Dr. Olga R. Povitzky of the laboratories of the New York Department of Health. The *H. influenzae* serum was of very high titer whereas the sera prepared with cultures of *H. influenzae suis* were of relatively low titers. All agglutination tests were conducted at 55°C. and readings were made after 2 hours at this temperature and refrigeration overnight. Cultures of *H. influenzae suis* tested were from widely separated sources and no two cultures were from the same strain of the disease (5). The results of these direct agglutination tests are given in Table III.

The data given in Table III indicate only one possibility that any two of the *H. influenzae suis* cultures are serologically identical. By use of the agglutinin absorption test it was possible to demonstrate that Cultures 652 and 660 were serologically identical. Conversely, by means of the agglutinin absorption test, it was possible to demonstrate that cultures of *H. influenzae suis* 451, 459, 652, and 660 which were agglutinated, but not to titer, by *H. influenzae* BIW were capable of removing no agglutinin for *H. influenzae* BIW.

Pathogenicity of H. influenzae suis for Laboratory Animals

As a rule *H. influenzae suis* is non-pathogenic for rabbits and can be administered intravenously to them in doses of 2 cc. of a heavy suspension of growth from chocolate agar without inducing any observable evidence of illness. However, such injections occasionally prove fatal in from 3 to 6 hours after injection.

One culture, when injected intraperitoneally, has been found to kill guinea pigs in from 18 to 24 hours. Two other cultures are completely non-pathogenic for guinea pigs.

TABLE IV

The Effect of Inoculating Swine with Pure Cultures of H. influenzae suis

Swine No.	Route of inoculation	Culture used			Clinical evidence of illness	Autopsy findings
		No.	Time since isolation	No. of transfers on media		
507	i.n.*	451	18 days	4	Suggestive of influenza	Suggestive of influenza
515	i.v.**	451	18 "	4	Moribund in 3 days	No respiratory pathology
739	"	451	17 mos.	86	Negative aside from transient temperture elevation	Not autopsied
591	i.n.	Mixture of 1928 cultures	All over 3 mos.	All 15 or more	Negative	Negative
590	"	"	All over 2 mos.	All 10 or more	"	"
588	"	11-T	12 days	6	"	"
648	"	15	16 "	5	"	"
651	"	660	17 "	5	"	"
826	"	791 + 794	14 and 17 days	7 and 8	Death in 2 days	Pneumonia
873	"	451 + 459	Over 2 yrs.	125 and 113	Negative	Negative
893	"	451 + 459	" 2 "	126 and 114	"	"
904	"	896	9 days	7	"	"
912	"	451	Over 2 yrs.	131	"	"
917	"	905	16 days	7	"	"
922	"	451	Over 2 yrs.	132	"	"

* i.n. = intranasally.

** i.v. = intravenously.

About 50 per cent of the white mice injected intraperitoneally with various strains of *H. influenzae suis* have died. The organism is non-pathogenic when injected intraperitoneally into white rats.

Pathogenicity of H. influenzae suis for Swine

In an attempt to determine the etiological relationship of the organism to swine influenza, thirteen swine were inoculated intranasally with cultures of *H. influenzae suis* from various sources and under cultivation for varying periods of time on artificial media. Two swine received the organism intravenously. The cultures used in these experiments were grown in defibrinated blood at the bases of plain agar slants and the usual dose administered was 2 cc. of this bloody condensation fluid. The results of these experiments are recorded in Table IV.

The data presented in Table IV indicate that *H. influenzae suis* was completely non-pathogenic for eleven of the thirteen swine to which it was administered intranasally. Given intravenously, a relatively recently isolated culture induced a severe illness. However, at autopsy no disease of the respiratory tract suggestive of swine influenza was encountered. There was an acute glomerular nephritis. An older culture given intravenously to another animal proved non-pathogenic.

Swine 507 and 826 are noteworthy in that both animals became ill after intranasal inoculation with *H. influenzae suis* and, in both, the picture presented could readily have been confused with swine influenza. Swine 507 was infected during the early part of the 1st year's work with the disease and at the time was considered to be a fairly typical although mild case of swine influenza. Since *H. influenzae suis* was obtained in pure primary culture from both the bronchial exudate and lung of this animal at autopsy, Koch's postulates had apparently been fulfilled for the organism, and the writers at first believed that it was the inciting agent of the disease. Subsequent work has indicated the mistaken nature of this view but no certain and acceptable explanation of this one experiment, provided Swine 507 actually had influenza, can be offered. It may be that the four transfers on artificial media to which the organism was subjected before its use in inoculating Swine 507 were insufficient to remove mechanically any accompanying virus. Swine 826 died 2 days after inoculation with *H. influenzae suis* and at autopsy presented pulmonary lesions very like those observed in fatal cases of swine influenza. The true pneumonia was limited to the cephalic lobes and, as in fatal swine influenza, the caudal lobes presented an intense bloody edema. *H. influenzae suis* was recovered in pure culture from the kidney, heart blood, and spleen, and was obtained in mixed culture from the lung and bronchial exudate. The disease which developed in Swine 826, however, was recognized as definitely different from swine influenza in that it was not contagious. A normal susceptible hog placed in the same unit soon after Swine 826 had been inoculated developed no disease. This is markedly inconsistent with the highly contagious nature of swine influenza. A possible explanation of the fatal pneumonia in Swine 826 is that this animal had been taken from its mother at birth, deprived of colostrum, and fed an artificial diet. The influence of such treatment on the course of an influenzal infection will be discussed more fully later.

The experiments recorded in Table IV seem sufficient to demonstrate that *H. influenzae suis* alone is not capable of inducing swine influenza even though it may under certain conditions be pathogenic for swine.

SUMMARY

1. A hemophilic bacillus has been regularly obtained in culture from the respiratory tract of a series of swine experimentally infected with swine influenza and from a small number of spontaneous field cases of the disease. It has not been observed in respiratory tract cultures from a group of swine free from influenza.
2. The cultural and morphological characters of the organism have been described and the name *Hemophilus influenzae* (variety *suis*) suggested. The organism exhibits marked serological diversity, since only two out of eight strains studied were serologically identical. It is usually non-pathogenic for rabbits and white rats, and irregularly pathogenic for white mice. One strain of the organism was pathogenic for guinea pigs while two others were not.
3. Eleven out of thirteen attempts to induce symptoms of disease in swine by intranasal inoculation with pure cultures of *H. influenzae suis* were entirely negative. The remaining two attempts which suggested a positive result have been discussed.
4. Attention has been called to the marked similarity which exists between non-indol-producing strains of *H. influenzae* and *H. influenzae suis.*

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EXPLANATION OF PLATE 35

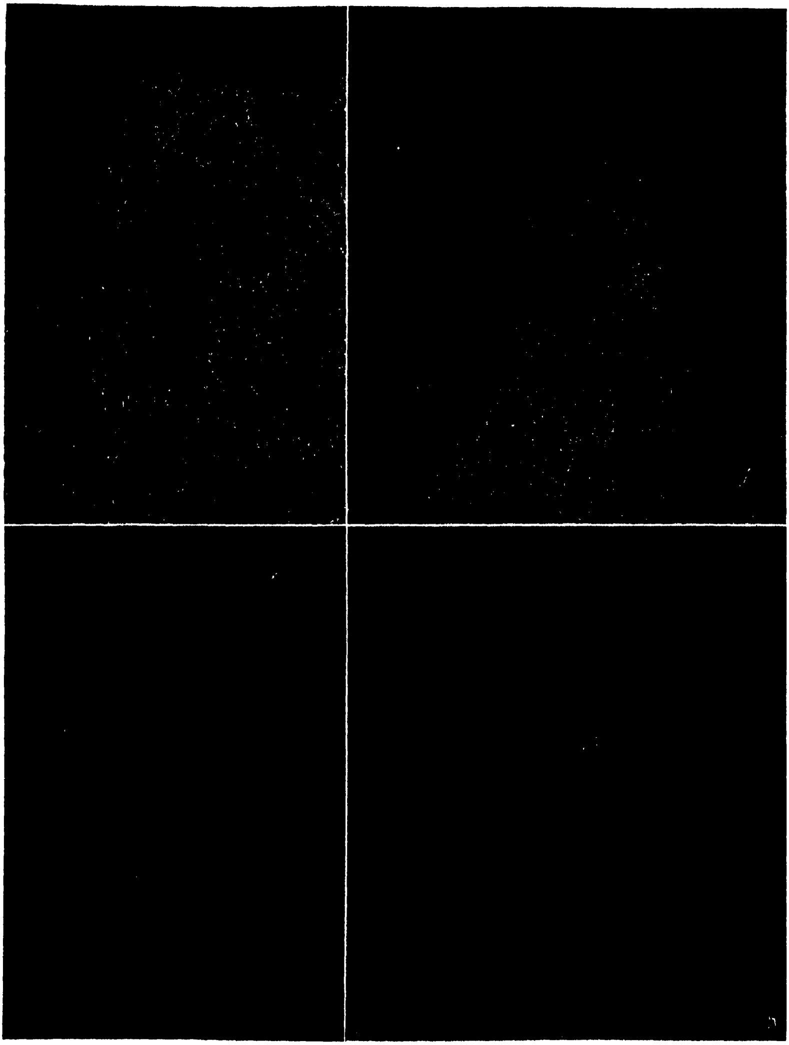
Films from cultures of *H. influenzae suis.* × 905.

FIG. 1. 24 hour chocolate agar culture of Strain 660 showing predominantly small bacillary forms. Alkaline methylene blue.

FIG. 2. 48 hour chocolate agar culture of Strain 913 showing predominantly long thread-like forms. Dilute carbolfuchsin.

FIG. 3. 72 hour blood broth culture of Strain 913 showing clumps of small coccoidal and bacillary forms. Dilute carbolfuchsin.

FIG. 4. 5 day blood broth culture of Strain 459 showing clumps of "giant" coccoidal and club-shaped forms. Alkaline methylene blue.



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SWINE INFLUENZA

III. FILTRATION EXPERIMENTS AND ETIOLOGY

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McBryde, Niles, and Moskey (1) made five attempts to pass the etiological agent of swine influenza through small Berkefeld or Mandler filters but were unable to reproduce the disease by dropping such filtrates into the nostrils of normal hogs. In two of these experiments unfiltered material failed to produce the disease in their control animals. Although these experiments are too few in number to be conclusive, they indicate that the causative agent of swine influenza is not a filtrable virus.

EXPERIMENTAL

Since the studies in filtration reported by McBryde and his coworkers were not conclusive, the question of the filtrability of the etiological agent of swine influenza has been reconsidered. The results of ten filtration experiments with infectious material from the two strains of the disease obtained in 1928 and the two secured in 1930 (2) were inconstant and confusing. In these preliminary experiments the writer was not then cognizant of the possible etiological relationship existing between *H. influenzae suis* (3) and a filtrable agent to be described.

Material for filtration was prepared as follows.

Diseased lung and bronchial lymph nodes were minced with sterile scissors and added to bronchial exudate. This mixture was ground with sand in a mortar. When it had been reduced to a pasty and fairly homogeneous consistency, a 10 to 20 per cent suspension was made by gradually adding sterile distilled water or infusion broth (pH 7.3). It was then shaken with glass beads in a flask for 10 to 15 minutes and centrifuged. The supernatant fluid was removed by pipette and if more than moderately turbid it was centrifuged again. 24 hour bouillon cultures of *B. prodigiosus* were used to test the efficiency of all filters, and filtrates cultured in 1 cc. amounts on plain agar slants containing defibrinated blood were incubated at 37°C. for 48 to 72 hours before examination for growth.

Swine receiving filtered material were placed in carefully sterilized isolation units where isolation precautions were taken. In certain experiments the control animal receiving unfiltered material and those receiving sterile filtrates were from the same source or even from the same litter.

Of the ten preliminary filtration experiments, three were at the time interpreted as negative, while in the remaining seven some evidence was obtained that the injected filtrate had contained an infectious agent. The disease induced by this filtrable infectious agent, however, was definitely not typical swine influenza and will be referred to hereafter as "filtrate disease."

Clinically the filtrate disease was much milder than swine influenza. In most cases there was no elevation in temperature, while in a few a fever temperature for 1 day was observed. This was at marked variance with the 4 to 6 day fevers seen in typical swine influenza. The usual symptoms shown by filtrate-inoculated swine were a moderate and transient apathy, some diminution in appetite for a period not exceeding 3 days, occasionally a slight cough, and, as in typical swine influenza (2), a moderate or quite marked leucopenia. The extreme prostration so common in swine influenzal infections was not seen. In some instances the disease was so mild that it almost escaped recognition altogether. On this account and in the light of experiments to be outlined later in this paper, it seems possible that in the three preliminary experiments considered as negative, infections were actually produced but so mild in character that they escaped recognition.

The lesions exhibited at autopsy were similar in kind but different in extent, as a rule, from those encountered in typical uncomplicated swine influenza (2). The cervical and bronchial lymph nodes were moderately enlarged and edematous and there was usually a scant to moderate amount of thick, tenacious mucous exudate in some of the smaller bronchi. The amount of pulmonary atelectasis exhibited by filtrate-infected swine varied from a scant amount in one or two of the upper lobes of the lung to an amount as extensive as that shown by mild cases of swine influenza. In all cases, however, the clinical picture was that of the filtrate disease.

It is significant that the filtrate disease was highly contagious and that the incubation period, like that of swine influenza, was about 4 days. In animals infected by contact with filtrate-infected swine the disease was clinically and pathologically identical to that induced by direct inoculation with the filtrate. In experiments to test the contagiousness of the filtrate disease, the normal animals were placed in the pens with inoculated swine after the appearance of symptoms of disease. This was usually 2 days following inoculation.

The filtration experiments just outlined indicated that infectious material from experimental cases of swine influenza contains an agent capable of passage through Berkefeld filters V and N and possessing pathogenic properties when administered intranasally.

H. influenzae suis, which was constantly encountered in culturing the respiratory tracts of animals with typical swine influenzal infections (3), was not found in similar cultures from animals with the mild filtrate disease.

Anaerobic cultures of seven filtrates of swine influenza infectious material in blood broth and in 5 per cent serum bouillon over sterile rabbit kidney have failed to show growth. Four of the seven filtrates thus cultured were tested by intranasal inoculation into swine and all were found capable of inducing the mild filtrate disease.

Intranasal Inoculations with Mixtures of the Filtrable Agent and H. influenzae suis

Since the only constant difference bacteriologically between the mild disease induced by the filtrable agent and typical spontaneous or experimental swine influenza lies in the absence of *H. influenzae suis* in the filtrate-infected swine, the combination of the organism and the filtrable agent may be essential for the production of the natural disease. Experiments were conducted in which swine were inoculated intranasally with cultures of *H. influenzae suis*, which had been under cultivation for a long time (over 2 years in most instances), mixed with Berkefeld filtrates of infectious material from experimental cases of swine influenza.

In these experiments the isolation and filtration practice outlined above was followed. The cultures of *H. influenzae suis* used were grown in defibrinated horse blood at the bases of plain agar slants in most instances for 24 hours. The undiluted blood culture was used in the inoculations and in all experiments the culture injected alone was identical with that mixed with filtrate before injection. The Berkefeld filtrate mixed with cultures of *H. influenzae suis* was identical with that injected alone in individual experiments. With the excep-

TABLE I

Effect of Inoculating Swine with Mixtures of Filtrable Agent and H. influenzae suis

Experiment No.	Infectous material from swine No.	Swine inoculated No.	Inoculated intranasally with	Clinical picture	Autopsy findings	*H. influenzae suis* in		Remarks
						Lung	Bronchial exudate or scrapings	
1	860 Strain 14 (1930) In infusion broth	859	10 cc. Berkefeld N filtrate	Mild filtrate disease	Very few	Absent	Absent	Illness extremely mild
		861	8 “ “ “ “ + 2 cc. culture HIS*	Typical and severe influenza	Influenzal pneumonia	Pure culture	Pure culture	More severe disease than control (871)
		871	10 cc. unfiltered suspension	Typical influenza	Typical	“ “	Mixed “	Control of unfiltered suspension
2	872 Strain 15 (1930) In infusion broth	875	4 “ Berkefeld N filtrate	Mild filtrate disease	Very few	Absent	Absent	Illness extremely mild
		874	4 “ “ “ “ + 2.5 cc. culture HIS	Typical influenza	Typical	Pure culture	Mixed culture	Disease about same severity as control (876)
		873	2.5 cc. culture HIS in 4 cc. infusion broth	No illness	Negative	Absent	“ “	Control of culture alone
		876	4 “ unfiltered suspension	Typical influenza	Typical	Mixed culture	Pure “	“ “ unfiltered suspension
3	878 Strain 15 (1930) In distilled water	894	7 “ Berkefeld N filtrate + 2 cc. sterile horse blood	Mild filtrate disease	Few	Absent	Absent	Scarcely recognizable illness
		897	Infected by contact with Swine 894	“ “ “	Not autopsied			“ “ “
		892	7 cc. Berkefeld N filtrate + 2 cc. culture HIS	Typical influenza	Typical	Mixed culture	Pure culture	Same severity as disease in control (895)
		896	Infected by contact with Swine 892	Very severe influenza	Influenzal pneumonia	Pure “	Mixed “	Moribund when killed
		893	2 cc. culture HIS in 7 cc. distilled water	No illness	Negative	Absent	“ “	Control of culture alone
		895	5 “ unfiltered suspension mixed with 10 cc. normal swine serum	Typical influenza	Typical	Mixed culture	Not cultured	“ “ unfiltered suspension
4	907 Strain 15 (1930) In infusion broth	911	4 cc. Berkefeld N filtrate	Mild filtrate disease	Few	Sterile	Sterile	Scarcely recognizable illness
		910	4 “ “ “ “ + 2 cc. culture HIS	“ influenza	Typical	“	Mixed culture	Same type of disease as control (913)
		915	Infected by contact with Swine 910	“ “	“	Pure culture	“ “	“ “ “ “ “ “ (913)
		912	2 cc. culture HIS in 4 cc. infusion broth	No illness	Negative	Absent	Pure “	Control of culture alone
		913	4 “ unfiltered suspension	Mild influenza	Typical but few	“	“ “	“ “ unfiltered suspension
5	918 Strain 15 (1930) In infusion broth	919	8 “ Berkefeld N filtrate**	“ filtrate disease	Not autopsied			Scarcely recognizable illness
		920	8 “ “ “ “	“ “ “	“ “			“ “ “
		921	8 “ “ “ “	“ “ “	“ “			“ “ “
		923	8 “ “ “ “ + 2 cc. culture HIS	Typical and severe influenza	Typical	Pure culture	“ “	More severe than disease of control (951)
		922	2 cc. culture HIS in 8 cc. infusion broth	No illness	Not autopsied			Control of culture alone
		951	8 “ unfiltered suspension	Typical influenza	Typical	“ “	“ “	“ “ unfiltered suspension

* HIS = *H. influenzae suis.*

** *B. prodigiosus* present in filtrate. *H. influenzae suis*, however, could not be demonstrated. All other filtrates recorded were sterile.

tion of the first experiment, all animals used in individual experiments were from the same source and in most instances litter mates. The results of these experiments are recorded in Table I.

All eight of the swine infected by inoculation with Berkefeld filtered infectious material or by contact with filtrate-infected swine developed only a mild disease. In some instances it was so slight as almost to escape recognition. None of the animals exhibited a febrile reaction. In 1 to 3 days after inoculation they appeared listless and apathetic for 2 or 3 days and there was some diminution in appetite. Those coming to autopsy showed enlarged and edematous cervical and bronchial lymph nodes, a small amount of tenacious mucous exudate in some of the smaller bronchi, and a scant scattered type of pulmonary atelectasis of one or more of the upper lobes of the lung.

The swine which were inoculated intranasally with pure cultures of *H. influenzae suis* were completely negative both clinically and at autopsy.

All the swine which received mixtures of the filtrable agent and *H. influenzae suis* developed a disease that was typical both clinically and at autopsy of swine influenza (4). Of the seven hogs infected either by direct inoculation with the filtrate-culture mixture or by contact with swine so infected, three developed typical swine influenza, and the disease was of about the same severity as that which developed in the control animals inoculated with unfiltered infectious material. Two others had a mild influenza, but in this instance the disease which developed in the control animal was also atypically mild. The remaining two swine developed exceptionally severe swine influenza and at autopsy both exhibited typical pneumonia. These two animals showed a more severe type of infection than did their controls infected by unfiltered material.

Swine recorded in Table I as "infected by contact" were not placed in the pens with the animals to whose disease they were to be exposed until the onset of illness in the inoculated animals.

Immunity Experiments

During the fall and winter months when swine influenza occurs, individual herds of hogs are occasionally reported to have swine influenza two or even three times during one season. In such instances the outbreaks follow one another at short intervals. The disease is very uncommon in animals (brood sows and boars) over 1 year of age on farms which suffered losses the previous year. This indicates the acquisition of immunity from the attack of the preceding year.

Our experience with the experimental disease indicates that the majority of animals are made immune by one attack of the disease. We have repeatedly placed freshly infected swine in the same units with convalescent animals without producing a second attack of the disease in the convalescent animal. This is in marked contrast to our experience with previously unexposed animals.

While the failure to develop the disease by contact is an indication of some degree of immunity, it is a less severe test than submitting the animals to actual reinoculation with infectious material. This has been done with six animals convalescent from typical swine influenza. These swine were inoculated intranasally with material, the infectiousness of which was controlled by parallel inoculation of a normal animal. The reinoculations were made at intervals varying from 15 to 24 days following the animal's first infection and 8 to 16 days after symptoms had disappeared. Of these animals, four were not reinfected, one developed a typical attack of swine influenza, and one developed a very abortive type.

One sample of convalescent serum has been tested for its neutralizing capacity.

The animal which furnished this serum, Swine 806, had undergone a typical, severe attack of swine influenza. After recovery it was exposed to four typical cases of the disease in sequence, remaining in the same pen with the infected swine from the time of inoculation until they were killed on the 4th day of disease. No illness resulted from any of the exposures. The serum was obtained 40 days after the initial inoculation and 16 days after the last exposure to a case of the disease. Strain 14 was the source of infectious material used either directly or by contact. The serum was tested against Strain 15 material as follows.

Swine 879 was inoculated intranasally with 5 cc. of Strain 15 material mixed with 10 cc. of convalescent Serum 806. The mixture stood at room temperature for 3 hours before use. No evidence of illness developed in this animal during a 10 day observation period. The control, Swine 895, inoculated intranasally with 5 cc. of the same infectious material mixed in the same manner with 10 cc. of normal swine serum, developed a typical swine influenza which was at autopsy found to be characteristic and extensive. Swine 879 after an observation period of 10 days was injected intranasally with 10 cc. of Strain 15 material. After a slightly prolonged latent period the animal developed typical swine influenza with a temperature reaching 41.5°C. At autopsy the disease was typical and extensive.

In this instance, then, serum of a hog that had undergone a typical swine influenza and that was demonstrably immune to reinfection by contact neutralized swine influenza material. No demonstrable immunity developed in the animal receiving the neutral mixture.

Storage of Infectious Material

Experiments to test the keeping qualities of the agents of swine influenza have been complicated by differences in the period of survival of the two components. Pieces of atelectatic lung and bronchial lymph nodes from one experimentally infected swine were stored for 15 to 33 days and from another swine for 15 and 41 days in 50 per cent glycerol before testing them for infectivity. They have been found capable of inducing only the mild filtrate disease typical in its course and at autopsy. With one exception *H. influenzae suis* has not been demonstrable in cultures from the respiratory tract of swine infected with this material. With infectious material frozen and dried by Swift's method (5) the disease induced by stored material was somewhat different. Material that had been stored for 34 days proved capable of inducing only the filtrate type of disease when inoculated into two susceptible swine and *H. influenzae suis* was not found in the respiratory tracts of these two animals at autopsy. However, another tube of this same material tested after 54 days' storage proved capable of inducing typical and rather severe swine influenza in which at autopsy *H. influenzae suis* was found in both the bronchial exudate and the atelectatic lung. It appears that the swine influenza virus is capable of storage in a dried state or in glycerol for at least 54 or 41 days, respectively, but that the bacterial component of the mixture is less resistant to such storage. The irregularity in the results obtained with dried infectious material may have been due to faulty freezing or drying of the particular tubes of dried material tested after 34 days' storage, for it is difficult to understand why *H. influenzae suis* should not survive freezing and drying. If it were desirable to preserve both factors the virus could be maintained in a dried state or in glycerol, while the organism could be kept under cultivation on artificial media and the two mixed before inoculation.

DISCUSSION

In a series of preliminary experiments to determine the pathogenic properties of bacteriologically sterile Berkefeld filtrates of infectious material from experimental cases of swine influenza, it has been possible in most cases to induce a definite but mild illness by the intranasal inoculation of swine with such filtrates. The disease thus induced has been transmissible by contact without altering its clinical or pathological characteristics. The possibility was thus eliminated that the filtrate disease is the result of inoculation with a toxic bacterial or tissue end-product or aggressin incapable of self-propagation in series. The observation that the illness which developed in animals exposed to cases of the filtrate disease does not differ in its clinical or pathological characteristics from that resulting from direct inoculation with the filtrable agent indicates that the mildness of the filtrate disease is not due to dilution of the inciting agent during filtration. Since the filtrate-induced disease has consistently been at variance with typical swine influenza, it was obvious that the disease induced by a filter-passing virus could not rightly be considered swine influenza. The impression gained after consideration of a series of these mild infections was that the disease both clinically and pathologically represented natural swine influenza in an incomplete form.

In the preceding paper (3) it was shown that a hemophilic bacillus, *H. influenzae suis*, was constantly demonstrable in the respiratory tracts of swine ill with influenza. It has been consistently absent from the respiratory tracts of swine ill with the filtrate disease. To test the possibility that swine influenza is the result of the two agents acting together, swine were inoculated intranasally with mixtures of the filtrable agent and *H. influenzae suis*. A disease typical of swine influenza in all clinical and pathological respects and indistinguishable from that induced by unfiltered infectious material resulted in all instances. Control animals receiving cultures of *H. influenzae suis* alone developed no evidence of illness and swine receiving the filtrable agent alone developed the mild filtrate disease. It seems permissible to interpret these experiments as indicating that swine influenza is due to a filtrable virus and *H. influenzae suis* acting together. Their mode of action is unknown although two possibilities are obvious:

The first possibility is that the pathological activities of the virus are such as to create a portal of entry for *H. influenzae suis* and to furnish a favorable medium in which it can multiply. Under such an assumption the virus serves merely as an entering wedge for the organism and the latter determines the clinical picture and pathology. There can be little doubt from the data presented in this and the preceding paper (3) that, in the presence of the swine influenza virus, *H. influenzae suis* possesses invasive powers which it completely lacks when administered alone.

The second possibility is that the virus is the important component in contributing to the pathology and perhaps also to the symptoms characterizing the clinical picture, and that *H. influenzae suis* increases to a marked degree the pathogenic properties of the virus and hence the severity of the resulting disease. In this respect, the influence of *H. influenzae suis* on the pathogenic properties of the swine influenza virus suggests the effect of certain tissue extracts on various viruses pointed out first by Duran-Reynals (6) and later amplified by Hoffman (7).

Whatever the relation of the virus and the organism in respect to the disease, the data presented indicate that they act together.

The hypothesis is not new that a disease may be induced by a bacterium and an invisible agent, not readily demonstrable alone. It applies most directly to diseases in which the suspected bacterial agent, while readily and uniformly isolated from cases of the disease, either is completely incapable of reproducing the infection or very rapidly loses its ability to do so under conditions of artificial cultivation. It is possible that such organisms do not become non-pathogenic because of rapid loss of virulence but because of the absence of an invisible inciting agent. The proven association of organisms in swine influenza furnishes a tangible and experimentally reproducible justification of this hypothesis.

A question which naturally arises is whether any organism other than *H. influenzae suis* can induce swine influenza in conjunction with the virus. In twenty-one infections of swine with virus alone none of the organisms comprising the normal bacterial flora of the respiratory tract have been capable of producing swine influenza. Also, the constant presence of *H. influenzae suis* in experimental infections induced by eight strains of swine influenza collected during three separate epizootics would seem sufficient to indicate that, if it is not the only organism capable of completing the etiological complex, it is at least the predominating one for the region from which our original material has been obtained.

It has been of interest to find in several of the fatal influenzal pneumonias that no organism other than *H. influenzae suis* could be cultivated from the pneumonic lungs, heart blood, or pleural and pericardial exudates. It is believed that in these cases, probably because of factors in the host itself, *H. influenzae suis* alone, or in conjunction with the virus, was endowed with invasive and pathogenic characters which in ordinary non-fatal influenzal infections it did not possess.

Since swine are subject to another filtrable virus disease, hog cholera, it has been essential to ascertain that the causative agent of swine influenza is not the hog cholera virus in a disguised or uncommon form. Our evidence indicating that the swine influenza virus is distinct from that of hog cholera may be summarized briefly as follows. Immunization against hog cholera affords no protection against swine influenza, while conversely animals recovered from swine influenza are still susceptible to hog cholera. The administration of hog cholera virus by way of the respiratory tract results in typical hog cholera with no tendency to produce pulmonary lesions suggestive of swine influenza. Convalescent swine influenza serum capable of completely neutralizing swine influenza virus does not neutralize hog cholera virus.

The clinical picture of swine influenza, characterized by fever, anorexia, extreme prostration, leucopenia, and evidence of respiratory involvement and of muscular tenderness, is strikingly suggestive of human epidemic influenza. The onset is sudden, the course short, and convalescence usually uneventful. Death, when it occurs, is the result of an edematous type of pneumonia. The pathology of non-fatal swine influenza, characterized as it is by an exudative bronchitis and extensive pulmonary atelectasis, cannot be compared with the findings in non-fatal human influenzal infections because of our lack of knowledge of the latter. Probably the most significant similarity concerns the predominant bacterium encountered in the two conditions; *H. influenzae suis* is indistinguishable morphologically and culturally from *H. influenzae*. The frequency with which *H. influenzae* has been encountered in careful bacteriological studies of human influenza parallels the frequency of occurrence of *H. influenzae suis* in swine influenza, and, as in the case of the latter organism, has suggested an etiological significance. Without drawing analogy too far, the irregularity in the outcome of the filtration experiments reported, especially by French and English investigators, in attempting to determine whether a filtrable virus causes human influenza, is very similar to the experience of the writer in the early filtration experiments with swine influenza. The preliminary obstacles encountered in studying the nature of the etiological factors in swine influenza have had much in common with those met by investigators of human influenza. A careful investigation would seem warranted of the possibility that Pfeiffer's bacillus and a filtrable agent act in concert to cause influenza in man.

SUMMARY AND CONCLUSIONS

1. It has been possible to demonstrate, in Berkefeld filtrates of infectious material from experimental cases of swine influenza, a virus which when administered intranasally to susceptible swine induced a mild, usually afebrile illness of short duration. The changes in the respiratory tract resembled those in swine influenza but were usually much less extensive. When the filtrable virus was mixed with pure cultures of *H. influenzae suis* and administered to swine a disease identical clinically and pathologically with swine influenza was induced. The data presented indicate that the filtrable virus of swine influenza and *H. influenzae suis* act in concert to produce swine influenza and that neither alone is capable of inducing the disease.

2. One attack of swine influenza usually renders an animal immune to reinfection. Blood serum from an animal made immune in this way neutralizes infectious material from swine influenza *in vitro*, as shown by the failure of the mixture to produce disease in a susceptible animal.

3. The virus can be stored in a dried state or in glycerol for several weeks at least. In one instance dried material apparently retained both the virus and *H. influenzae suis* in viable form for a period of 54 days.

4. Fatal cases of experimental swine influenza have been observed

in which *H. influenzae suis* was the only organism that could be cultivated from the respiratory tract.

5. Attention has been called to some features of marked similarity between epizootic swine influenza and epidemic influenza in man.

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STUDIES ON MILK SECRETION

THE INFLUENCE OF INANITION

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Milk secretion may be looked upon as a two-step process. The first step consists in the passage of the materials for the formation of milk from the blood stream into the gland cells. In the second step these materials are organized by the gland cells into the constituents of milk, when they are then passed into the alveolar spaces of the udder. The question arises as to what may be the motivating causes which bring about these changes.

The early contributions to this subject were largely those made by the histologist studying the changes which he observed in the tissue of the mammary glands. These histological observations have been markedly influenced in their interpretation by the supposed modes of secretion of two other types of glands, the sebaceous and the salivary. This work led to three major hypotheses to account for milk secretion by the mammary glands:

1. The cells of the glands break loose bodily and disintegrate in the alveoli to form the milk solids.
2. A portion of the cells toward the alveoli become loaded with solids, are broken from their base, and disintegrate to form the constituents of milk.
3. The cells of the mammary glands secrete the materials of the milk solids without the cells breaking down.

The first theory has largely been discredited because as Heidenhain (1)[1] pointed out this theory would call for a cell destruction and replacement of as high as five times the number of cells in the udder in a single day; a rate of cell replacement which was not observed.

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[1] The literature citations are given in the paper following.

The second theory, suggested by Langer and supported by Heidenhain (1), Steinhaus (2), and Brouha (3), lays its foundation on histological evidence. The gland cells are represented as lengthening out into the lumen of the alveoli. The projecting ends of these cells become loaded with nutrients similar to those of the milk solids, these projecting ends disintegrate and allow the solids to escape. The basal portion of the cell, including the nucleus, is left to rebuild the cell and repeat the process.

The third theory lays its stress on analogy with the other secretory glands. In support of this thesis Bertkau (4) says that the disintegration of the udder cells which other observers have claimed visible in mammary gland tissue is due to imperfect fixation and that when the tissue is properly fixed no necrobiosis of any kind appears. This theory is further supported by the study of the individual constituents of milk and their variations during the day and the lactation period (Gowen (5)).

As indicated above, milk secretion may be looked upon as a two-step process,—the passage of the precursors of milk from the blood into the secreting cells and their subsequent passage from these cells into the milk cisterns. The first two theories cited only visualize the last phase of this process. The third theory accounts for the whole process if we assume with Simms (6) that the constituents on the two sides of the cell membranes have unlike affinities for salts, etc., or secondly are differentially permeable.

As blood is the common carrier for the materials utilized in metabolism, it is natural to turn to it as the source of the precursors of milk solids. Thus far, the most fruitful method of analyzing what these precursors of the milk solids may be, has been the comparing of the composition of the blood which is entering the mammary gland with the composition of that coming away from it. This method of approach, first devised by Kaufmann and Magne (7), has shown that in all probability dextrose is the source of lactose in milk. The observations on which this conclusion is based have been substantiated by Cary (8) and also by the present writers (unpublished).

Meigs, Blatherwick, and Cary (9) have utilized the same methods to indicate the origin of milk fat. The reasoning is based on their experimental observations as follows:

The blood on entering the udder carries more phosphatides than that leaving the udder. The milk entering the udder carries less inorganic phosphate than that leaving the udder. From these observations and the difference in the composition of milk compared to that of blood with regard to phosphorus, they draw the conclusion that the mammary gland in butter fat secretion takes phosphatides from milk, converts them into fat, and returns as inorganic phosphorus the excess of this element derived in breaking down and synthesizing the milk fat from the phosphatide.

Cary following the same line of approach showed that the blood entering the udder contains more amino nitrogen than the blood coming away from the udder. From this fact and the work on metabolism of proteins he draws the conclusion that the proteins of milk are derived from the amino acids of blood.

In studying the composition of the fat deposited in the udders of cows in milk secretion, as contrasted with the udders of such cows in the dry period, the fats found in the actively secreting udder were found to be partly metabolized toward the characteristic fats of milk. The fat in the dry udder, on the other hand, does not show this step even though it contains proportionately much more fat than the actively secreting udder. The secretion of milk-fat appears as a distinct two-step process. The blood brings to the udder materials for deposition in the udder of fats comparable to other body fats. These fats are then converted by the udder, somewhat in advance of their need for milk secretion, into the fats characteristic of milk (Gowen and Tobey (10), Peterson, Palmer, and Eckles (11)).

These hypotheses and the facts just cited, however imperfect they may be, furnish an important approach to the problem of the mechanism of milk secretion. They illustrate, however, the desirability of other methods of attack. In the summers of 1925, 1926, and 1927, the writers attempted such an approach.

In view of the fact that most of the original constituents of milk find their origin in the blood stream, it would follow that if any means of altering the chemical characteristics of the blood stream could be found it would thereby be expected to affect the characteristics of the milk secreted. The particular type of alteration observed in the blood compared with the changes noted in the milk would furnish some idea as to the precursors of the constituents of milk as they are found in the blood. Furthermore, it seemed probable that the changes brought about by altering the blood stream would affect the physical characteristics of the blood stream, such as the osmotic pressures, etc., and by the subsequent alterations in the composition of the milk so produced furnish important facts on the mechanism by which it is normally secreted.

Four means of altering the characteristics of the blood seemed open to us. The first was the change brought about in the blood stream by complete inanition; the second method consisted in the introduction of the hormone of the parathyroid gland with its effect on the calcium of the blood; the third consisted in the introduction of insulin into the animal with the attendant lowering of the blood sugar and the other changes in blood composition; and lastly, it seemed desirable to check the effect of phloridzin on cow's milk rather than on goat's, as had been done by others. The experiment on inanition was also regarded as a control of the experiment on the use of insulin, as at that time it was believed that insulin would not produce its effect without inanition accompanying it. This experiment may be considered first. It may also be combined with the experiment on the action of the parathyroid hormone plus starvation, as the data on this experiment indicate that it is the starvation which produced the observed effects; the parathyroid hormone being without observed effect.

Material and Methods

Our experiment on the inheritance of milk production (11 etc.) included eight cows which were available for the work herein described. The cows ranged in production up to 40 pounds of milk a day and included animals with both high and low butter fat percentages in their normal milk. The animals were all in good condition for milk production, had no tuberculosis, as proved by their clinical tests and autopsy. (The herd is accredited.) The animals were maintained on a ration which by present standards was more than adequate for their milk production. In the initial period the animals passed through the same routine as that which followed for the experimental periods; they were milked, watered, cleaned and, if given food, fed at the same time as during the experimental period.

The first effort was directed toward determining what the normal variation in the milk production at these times really was and the variations in the composition of the milk so produced. In the chart the initial daily milking periods, before the experiment was commenced, represent quite accurately the true milk secretion of the given cow in this period of lactation fed on a production ration.

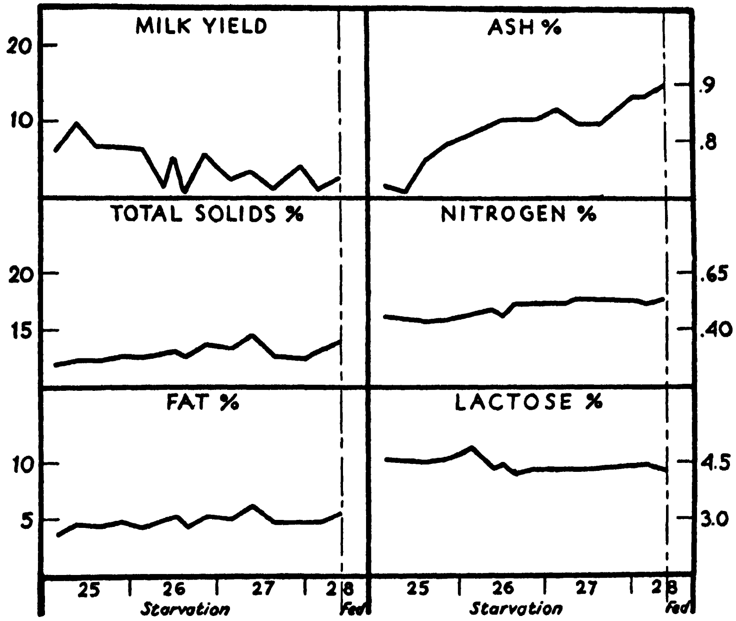

FIG. 1. Cow 47, age 8 years 11 months, 3 months in lactation. Food completely withheld; water given freely. Throughout the amount of material is plotted against time in days.

The total solids, fat, nitrogen, ash, and lactose, were determined by the methods approved by the Association of Official Agricultural Chemists. The calcium was determined by Collip's modification of the Kramer and Tisdall method. Phosphorus was determined by the method of Benedict. Folin's method of blood sugar determination was used and Benedict's method for estimating sugar in urine. The calcium and sugar of the urine appeared in such small amounts with no apparent relation to the other observations that they are omitted from the discussion. In the first experiments on two cows, 47 and 141, the milk calcium and phosphorus and blood calcium and dextrose

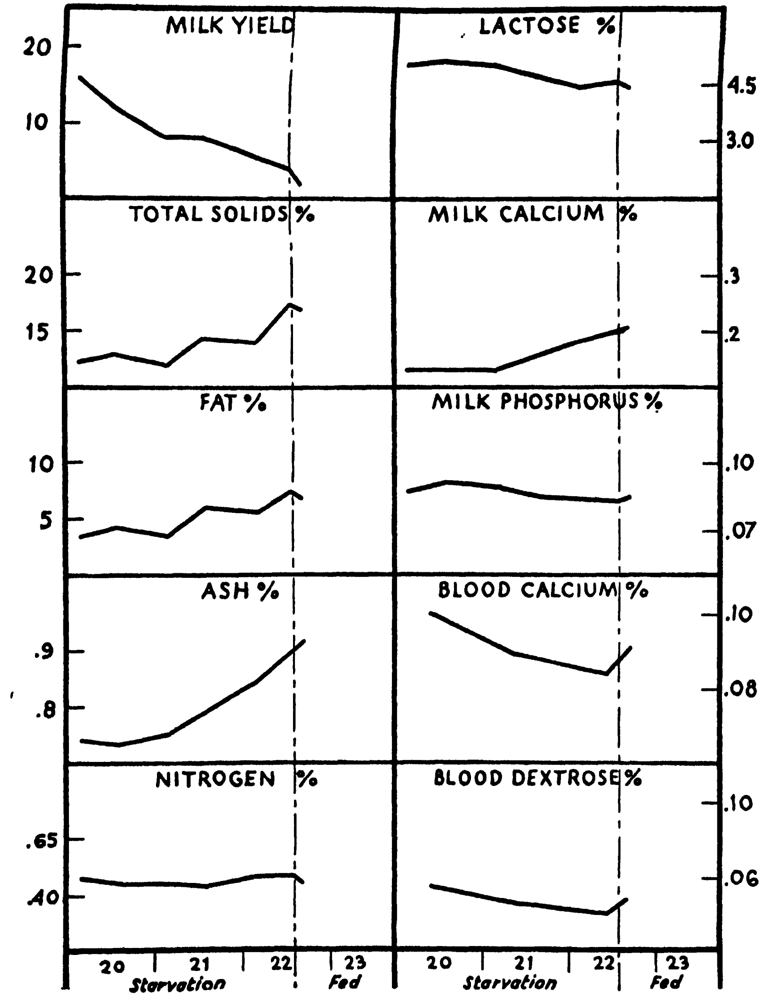

FIG. 2. Cow 47, age 9 years 11 months, 4 months in lactation. Food completely withheld; water given freely.

were not determined. The milking periods were in general at 5 o'clock in the morning and 3 o'clock in the afternoon. In certain cases when closer intervals were desired the milking periods were 5 and 11 o'clock in the morning and 5 in the afternoon and 11 o'clock in the evening.

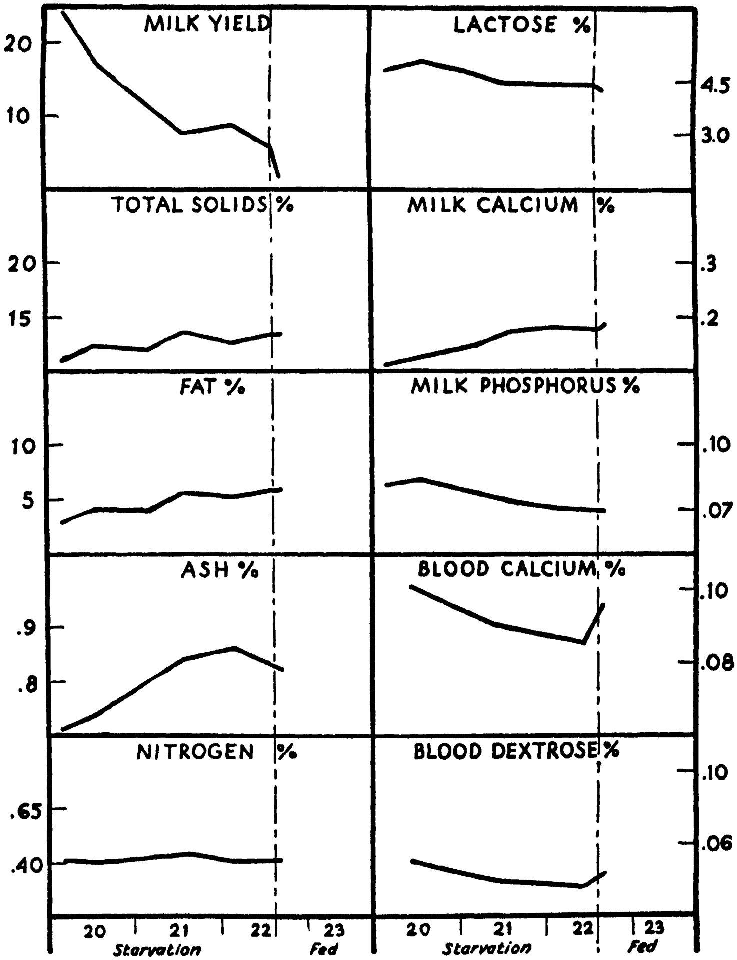

FIG. 3. Cow 52, age 9 years 8 months, 2 months in lactation. Food completely withheld; water given freely.

The Data

The milk yields and percentage composition of the milk and blood are presented chronologically for each cow in Graphs 1 to 13.

The figures are divided into two series of charts, on the left and right hand sides of the page. The upper left hand chart gives the milk yield in pounds plotted against time—duration of starvation. The charts which follow are per cent total solids, fat, ash, and nitrogen of the milk produced; all plotted against duration of inanition. The upper right hand chart is per cent of lactose. Following in sequence

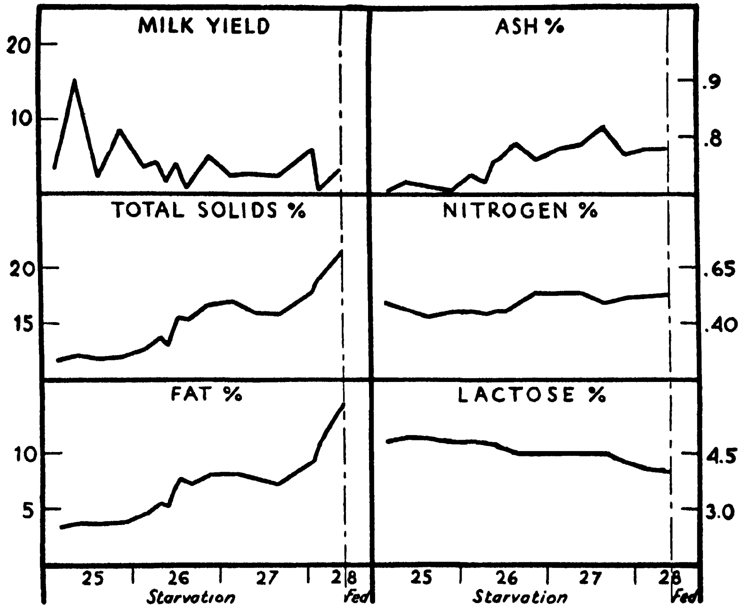

Fig. 4. Cow 141, age 5 years 0 months, 4 months in lactation. Food completely withheld; water given freely.

down the page are per cent of milk calcium, milk phosphorus, blood calcium, and blood dextrose; each plotted against the same chronological scale.

DISCUSSION

Relation between Inanition and Milk Secretion

Figs. 1 to 8, inclusive, bring out certain facts which, while not new to the literature of inanition have as yet not been explained. The cows all exhibit a rapid drop in milk yield almost from commencement of the inanition period. The rate of this decrease is greater during the first period of inanition, tending to tail off as inanition progresses

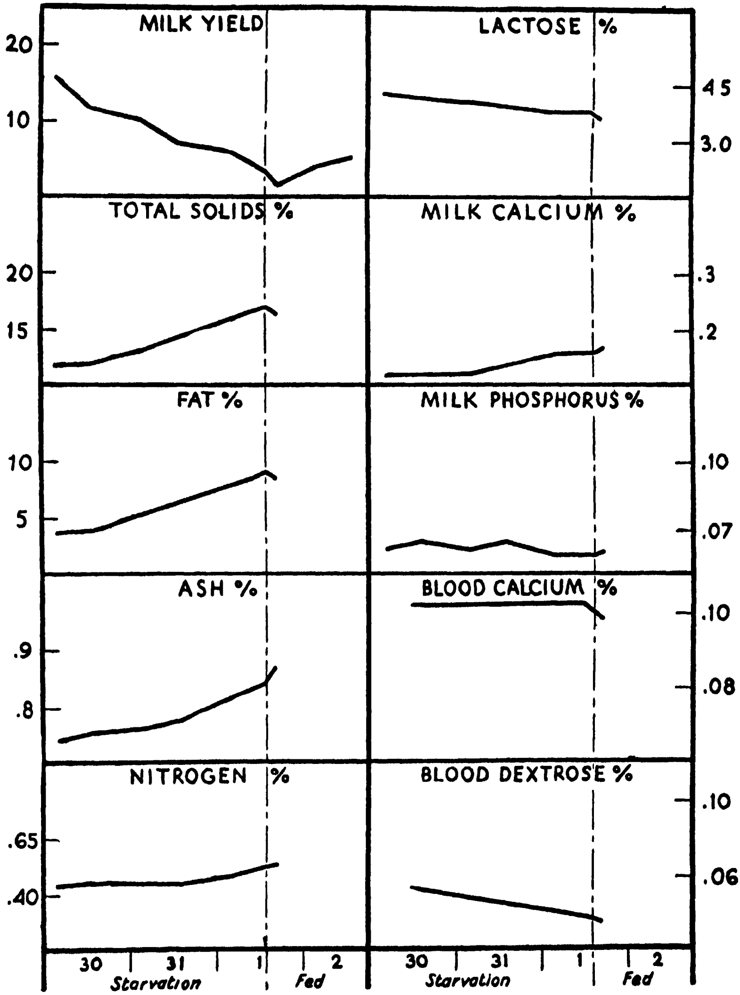

Fig. 5. Cow 141, age 6 years 0 months, 4 months in lactation. Food completely withheld; water given freely.

to an asymptotic value of zero milk producton. As the milk production decreases the percentage composition of the solids in the milk tends to increase rapidly to values which may be two times as great as those ordinarily found within milk. The average increase for the

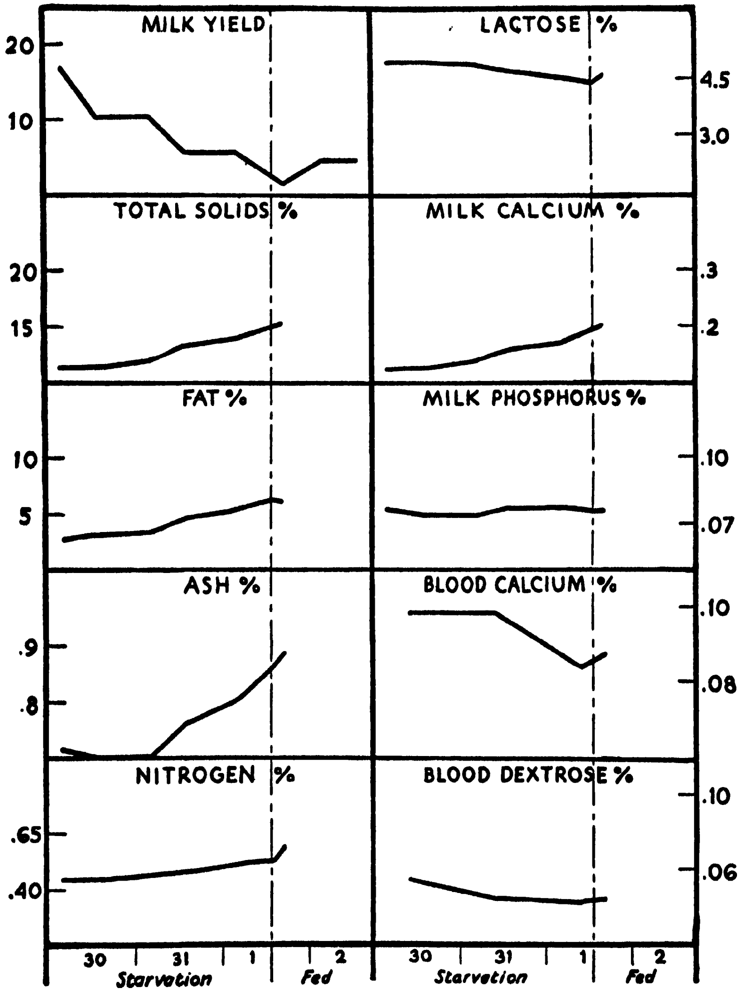

FIG. 6. Cow 212, age 10 years 0 months, 12 months in lactation. Food completely withheld; water given freely.

duration of the starvation period is 1.3 times the customary total solids per cent of these milks. This increase in total solids is very largely accounted for by the increase in the percentage composition of the butter fat. The butter fat may reach a percentage of 2.5 times

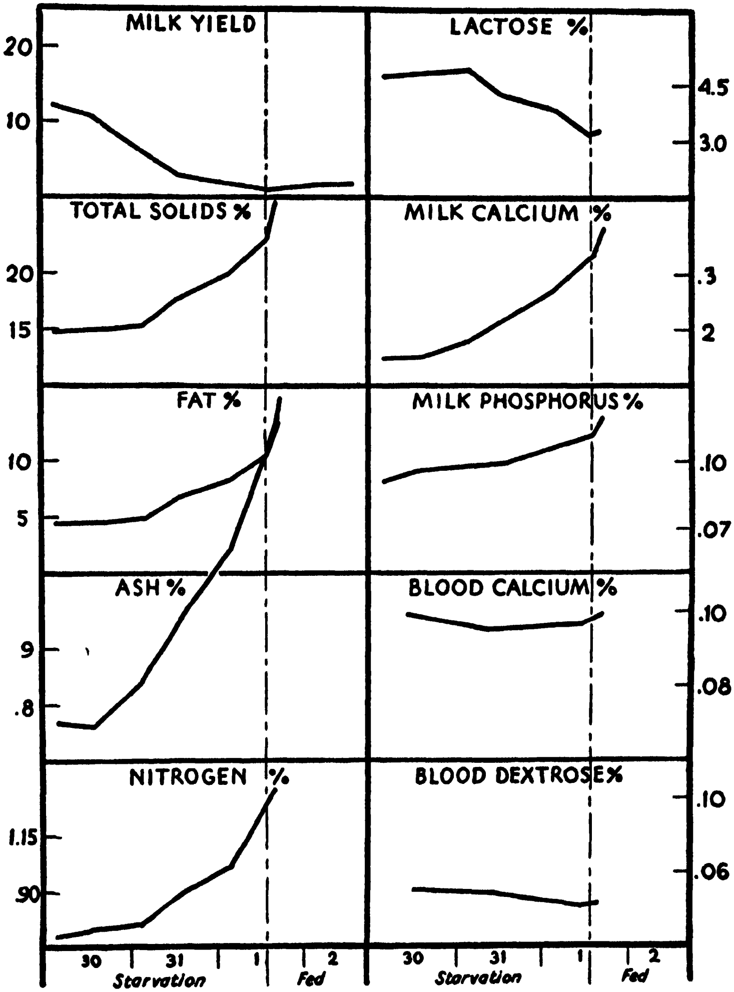

FIG. 7. Cow 213, age 5 years 10 months, 1 month in lactation. Food completely withheld; water given freely.

its normal value. The average increase for the duration of these experiments is 1.9 times the normal value. The ash content of the milk also increases in a marked degree. In one case this increase amounted to 1.7 times the ordinary ash content of the milk. The average gain was about 1.2 times the normal ash per cent for milk. The percentage content of the nitrogen rose but slightly during the inanition period for seven of the cows. For the eighth cow it changed

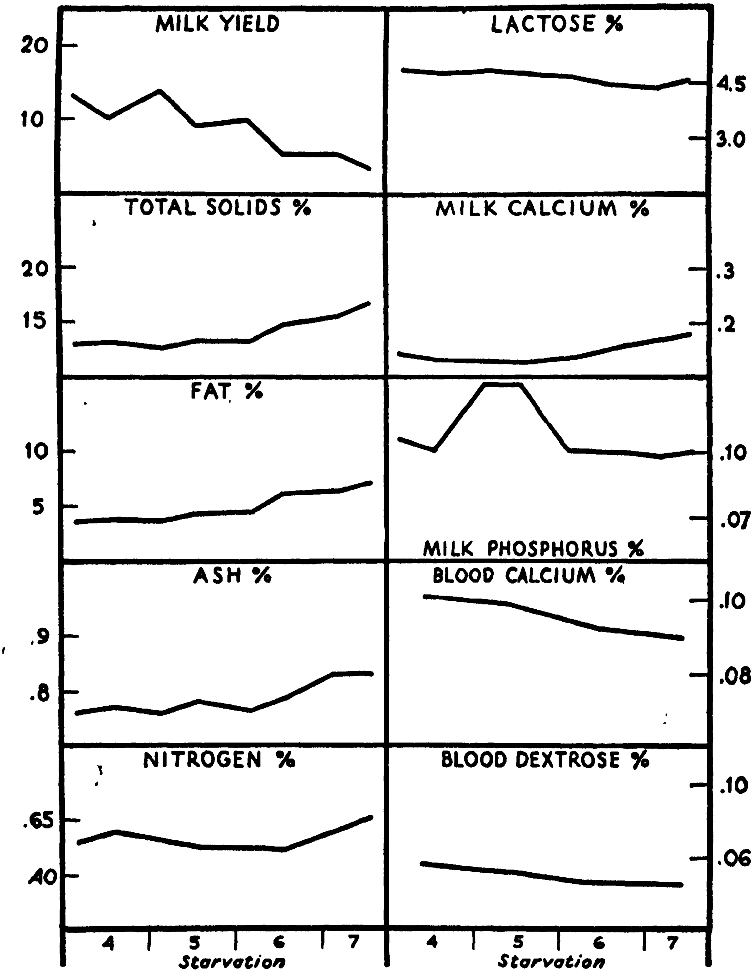

Fig. 8. Cow 215, age 6 years 7 months, 8 months in lactation. Food completely withheld; water given freely.

markedly. The nitrogen content of this cow's milk rose to nearly twice its normal value.

The lactose percentage, in contradistinction to the other materials of the milk, showed a distinct decrease in its percentage as the duration of the starvation period increased. This decrease on the average amounted to about one-tenth of the amount of lactose ordinarily found. The milk calcium increased to a somewhat greater extent than did the ash percentage, showing that the ash constituents probably changed in a manner out of proportion to their ordinary content in the ash of milk. The phosphorus content of the milk behaved irregularly but on the whole took a neutral trend throughout the period of inanition.

The blood calcium was found to decrease slightly during the period of inanition. As starvation progressed, the blood sugar was found to decrease.

These results agree with those of Overman and Wright (12) in all essential particulars. For their experiment three cows were available and the inanition period was of longer duration than that noted here. The longer duration of starvation simply extended the effects. The milk yield was reduced to a somewhat lower point, the total solids, fat, protein, and ash, were increased to higher percentages, whereas the lactose was decreased to a markedly lower amount. They do not consider the blood changes. Before considering the mechanism by which these changes are brought about, the data for starvation plus the injection of the parathyroid hormone may be noted. The parathyroid hormone was thought to offer a means of altering the composition of the blood of the cow for another constituent, the calcium. As this constituent plays a large rôle in milk secretion, being particularly associated with the casein, it seemed worth while to try the hormone's effect on milk secretion. With the information which had been gained from the use of insulin, it seemed that the amounts which would be necessary to produce an effect would be relatively high. 200 or 400 units were used at an injection; the material being injected directly into the jugular vein. The parathyroid hormone was the best available. We are indebted to Eli Lilly and Company for it. Unfortunately, the cow takes so much of this hormone that even 1000 units, over the relatively short time of two days, did not alter the composition of the cow's blood. This was true for both sets of animals; one set with the hormone accompanied by complete inanition, the other set with the hormone accompanied by feeding the normal ration. The

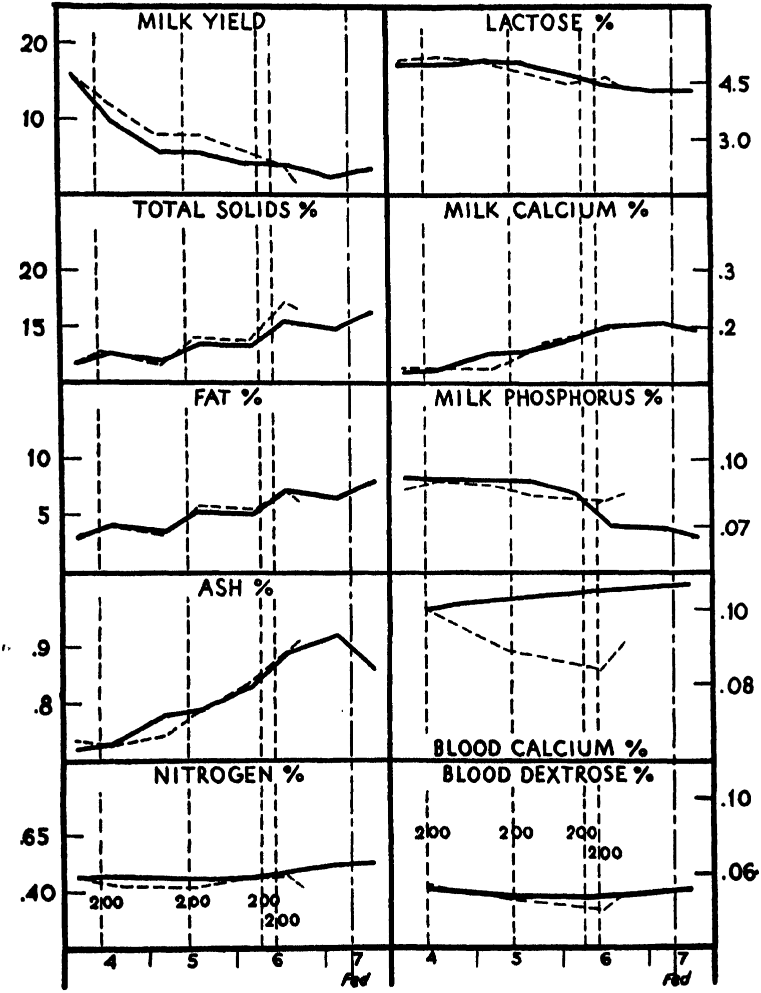

FIG. 9. Cow 47, parathyroid and complete inanition. The vertical dashed lines on the chart show the amount and time that the parathyroid hormone was injected. The solid line represents the data of this experiment,—the dotted line the data for the same cow on inanition alone.

results are, therefore, to be interpreted in the light of starvation experiments rather than as effects produced on the calcium of the blood by the parathyroid hormone. The facts obtained, aside from their own intrinsic interest, are of considerable importance as comparative

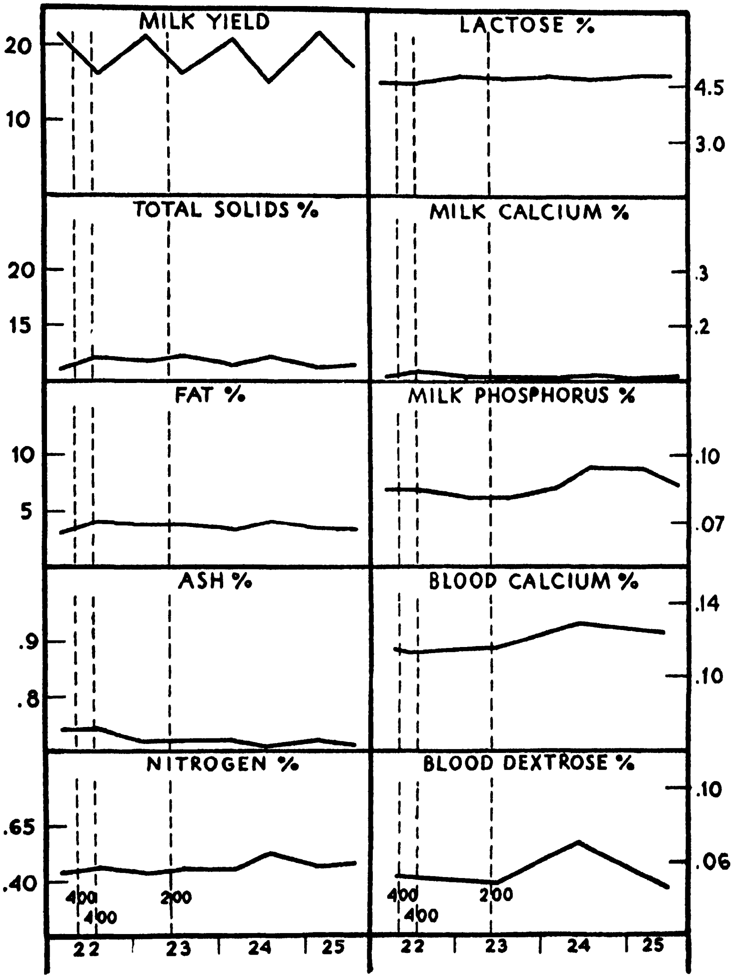

FIG. 10. Cow 52, parathyroid hormone and full ration throughout experiment. The amount of the parathyroid is shown by the vertical dashed lines on the chart at the time when the injections took place.

material for the insulin experiments which follow. The charts in Figs. 9, 10, and 11 present this material. Cow 52 received a full

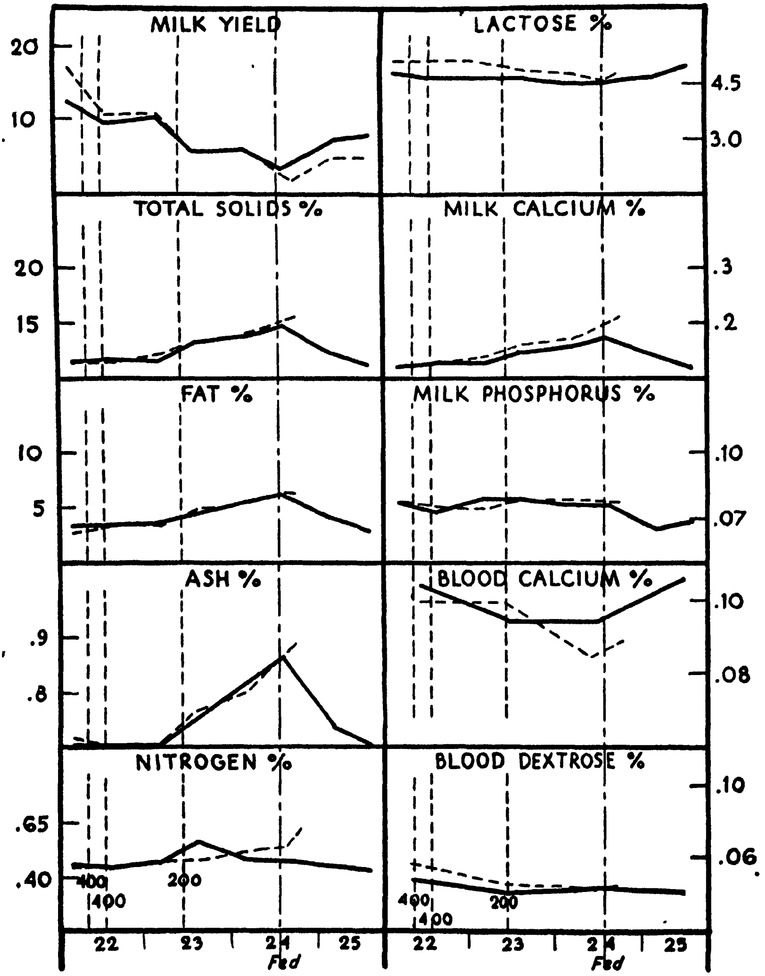

FIG. 11. Cow 212, parathyroid and complete inanition. The vertical dotted lines on the chart show the number of units of parathyroid hormone at the time when they were injected.

ration throughout the experiment. Cows 47 and 212 were under complete inanition. The times and amounts of the parathyroid hormone injected are shown on the vertical dashed lines on the chart. As will be noted in the earlier experiments, these cows were used for previous complete inanition experiments. The results for these earlier experiments are shown on the dotted lines.

Fig. 10 pictures milk secretion by a cow receiving an adequate ration. Attention is called to the relative steadiness in the percentage composition of all of the constituents of the milk, maintaining as they do their normal level throughout the entire experiment. This chart should be contrasted with the other cows under starvation. The graph for Cow 52 contrasts sharply with the one obtained in the starvation experiment. The chart for Cow 52 should also be compared with the subsequent one for Cow 47 when she received insulin and a full ration. The insulin treatment of Cow 47, in contradistinction to the parathyroid treatment of Cow 52 and despite her receiving a full ration, caused all of the constituents of the milk and the quantity of milk produced to show a definite and marked effect of the insulin. It is evident that the results for starvation and insulin are to be accounted for by their effect on milk secretion and not to any extraneous variables.

Figs. 9 and 11, for Cows 47 and 212, show simply the characteristic picture of the effects of starvation on milk production. The milk yields drop; the total solids, butter fat percentage, ash percentage, and calcium in the milk all rise. The nitrogen per cent remains fairly constant. The lactose per cent contrasts with the other elements of milk in that it tends to seek lower levels than are normal. Accompanying this change is a reduction in the dextrose content of the blood during the inanition period. On feeding the animals the different items return to normal.

What, it may be asked, is the mechanism by which these changes are brought about? The most noticeable fact is the difference in the behavior of the lactose and the behavior of the other constituents of the milk. In the case of the lactose the percentage composition decreases while in the case of the other constituents the percentage composition increases. As indicated earlier from the results of the composition of blood before and after it had passed through the udder the conclusion is drawn that the dextrose of the blood is extracted from it as it passes through the udder and is organized into the lactose of the milk by the udder cells. If this conclusion is correct, it would be expected that if the dextrose of the blood is decreased the lactose of the milk would likewise be decreased if it has no other origin. The fact that this is just what happens in these experiments furnishes further and independent proof for the correctness of the former conclusion; namely, that the lactose of milk is derived from the dextrose of the blood.

This change of the dextrose of the blood and the concordant change in the lactose of the milk is, we believe, a key to the explanation of these results. It has been known for a long time that the milk of individual cows is relatively stable for their lactose, ash, and protein content and less stable for their fat percentage (Eckles and Shaw (13) and others). It is likewise known that under similar normal conditions the constitution of the blood of an animal is relatively stable in its composition for the dextrose, salt, and protein content. The osmotic pressure of the blood and of the milk under normal conditions is essentially the same when the fat, which is not in the same phase as the rest of the system, is disregarded (Jackson and Rothera (14), and Davidson (15)). The osmotic pressures of milk are very largely determined by their lactose content, whereas the osmotic pressures of blood are relatively little determined by their sugar content; the large factor being the salt. It would therefore follow that if the osmotic pressures of the blood and milk are to be kept in constant ratio the milk of a lower lactose content would have to increase its content of salts in a rather pronounced degree to make up for this deficiency of lactose, since the salts are the only other large element capable of contributing to the osmotic pressures of the milk.

The osmotic pressures of the milk may be computed from the millimolar concentration of the lactose and ash found in the milk. A difficulty arises in that the ash is composed of six major osmotically active elements, potassium, sodium, calcium, magnesium, phosphorus, and chlorine, with a little sulfur and iron. The exact analysis of only two of these was obtained, namely calcium and phosphorus. The variation of these two elements indicates that the proportions of all six elements may be assumed as roughly the same as in normal milk ash for the purpose of calculating the osmotic pressure. As pointed out earlier, a decrease in the lactose should materially decrease the osmotic pressure unless the salt content increases to compensate.

Since the latter actually takes place, it is of interest to note the changes in osmotic pressure of milk which result from continued inanition. These are given in Fig. 12.

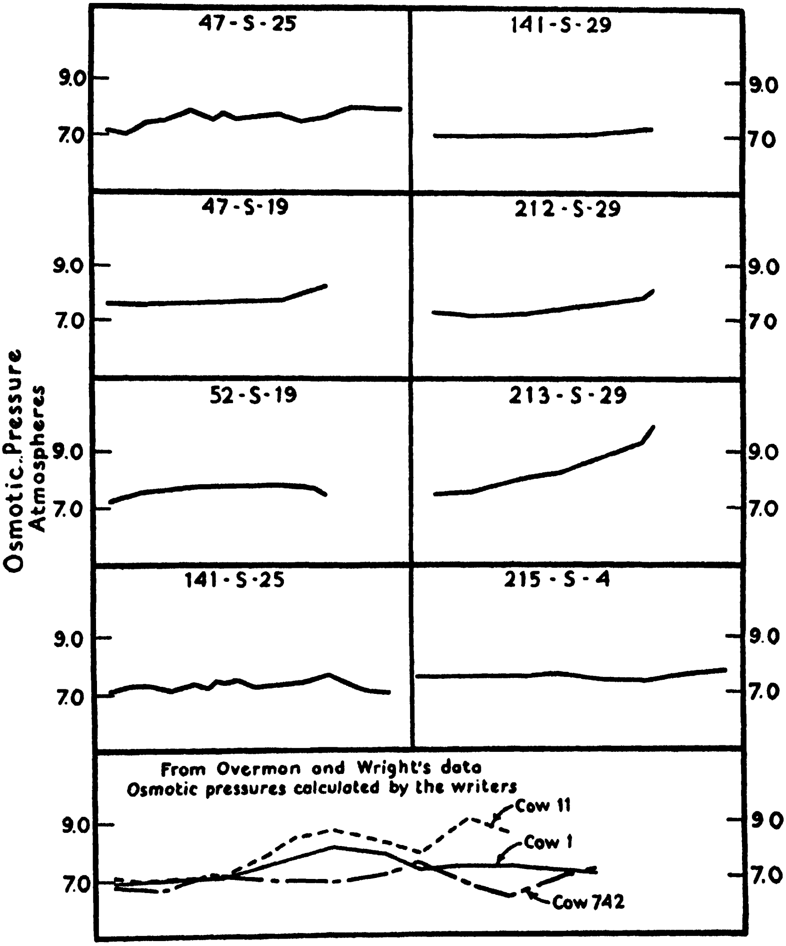

FIG. 12. Relation between the calculated osmotic pressure of milk determined from the proteins, ash, and lactose, in inanition.

Before considering the facts brought out by Fig. 12, it is well to note the changes in the blood during starvation. Mayer (16) has shown that the osmotic pressure of the blood of fasting dogs increases with the progress of inanition. Tria (17), working with both dogs and rabbits, obtained like results using the method of depressed freezing point. Polányi's (18) experiments on dogs lead to the same conclusion. It therefore seems to be rather conclusively proved that there is an increase in osmotic pressure of blood serum during the progress of inanition. As Morgulis (19) points out this change is apparently due to a greater ash content in the relatively higher proportion of the water soluble salt, chiefly sodium chloride.

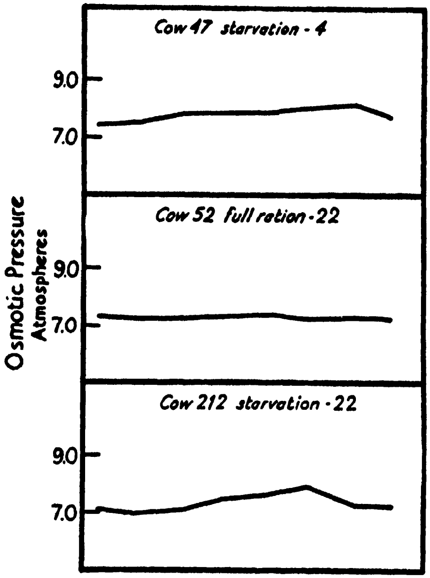

FIG. 13. Calculated osmotic pressures of milks from cows treated with parathyroid hormone.

From Fig. 12 it will be observed that the cows on starvation show a slight general increase in the osmotic pressures of their milk. In only one cow, Cow 213, was this increase marked. This cow is the animal which showed the greatest decrease in lactose in her milk. This decrease was accompanied by the greatest increase in the content of salt and nitrogen. The calcium content of the salt increased 2.4 times while the salts as a whole only increased 1.7 times indicating that the apparent increase in calculated osmotic pressure may be fictitious for Cow 213 since the calcium salts very possibly combine in the proteins.

If we calculate the osmotic pressures of the milk in the experiment of Overman and Wright, we find that for their three cows they also have an increase in the osmotic pressures of these milks. These changes were most pronounced in cows which were nearing the end of their lactation; whereas for the cow beginning her lactation the osmotic pressure did not rise to any extent.

The osmotic pressures of Cows 47, 52, and 212, treated with parathyroid are shown in Fig. 13. Cow 52 shows that a cow with a normal ration has practically a constant osmotic pressure. The osmotic pressures of the cows under starvation, 47 and 212, rise somewhat toward the last of the experiment, due no doubt to the change in osmotic pressure of the blood during inanition.

The decrease in the lactose of milk results in the necessity of a marked increase in the salt content of the milk if the osmotic pressure of this milk is to remain the same. This increase is actually observed and the osmotic pressures of milk, as determined from its composition, follow those which are to be expected of blood with progressive starvation. We may therefore conclude that the changes in the composition of milk during inanition are the results of the changes in the composition of the blood.

The behavior of the butter fat percentage in contrast to the percentages of the other constituents is of interest in the light of the results of the writers (10) in indicating that the fat of butter fat was first deposited in the udder from the blood and then metabolized into the characteristic fat of milk. The rate of metabolism of this fat is such that there appears to be stored in the udder in advance of milk secretion a limited amount of reserve fat which is equivalent to butter fat. If this is the case, the amount of this fat released into the alveolar spaces of the udder should be somewhat independent of the other constituents of milk. If the proportionate rate of change of the butter fat be examined it is found to increase from 1½ to 2½ times its initial amount as starvation progresses during the experiment. The ash increases to the extent of only 1.1 to 1.3 times the original amount. The nitrogen, on the whole, changes very little. The lactose decreases. These facts indicate that the butter fat is to some extent independent of the other constituents of the milk. This result is accordingly in agreement with the previous work. It might also be added that the more or less independent movement of the butter fat as contrasted with the other solids is seen in comparing milks of different breeds with differences in fat percentage.

SUMMARY

In this paper data are presented on cows receiving no food but having access to all of the water which they wished. The yield and composition of the milk were determined at various times during the periods of starvation. The composition of the milk showed changes which were progressive in the sense that they followed a definite course. They were characterized by a marked lowering in the amount of milk produced, by an increase in the total solids (chiefly an increase in the percentage of fat and ash, with a slight increase in proteins), and by a pronounced decrease in the lactose. The decrease in lactose corresponded with a decrease in the dextrose content of the blood, thereby supporting the conclusion that the lactose of milk has as its precursor dextrose of the blood. All the changes in milk composition during starvation can be directly related to the simultaneous changes in the blood.

The following companion paper on insulin and phloridzin as they affect milk secretion further develops these hypotheses.

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ON THE MECHANISM OF MILK SECRETION

THE INFLUENCE OF INSULIN AND PHLORIDZIN

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Insulin

The introduction of insulin into the blood stream of dairy cattle should markedly lower the blood sugar and bring about the characteristic changes attendant thereto if the hypotheses developed in the previous paper are correct. In the initial experiment of 1924 we were kindly supplied insulin by Armour and Company, through the courtesy of Dr. Frederic Fenger. In all of the subsequent experiments, in fact all of those reported here, we have been supplied insulin by Eli Lilly and Company, through the courtesy of their research director, Dr. George H. Clowes.

The initial experiments were devoted to finding the approximate dose which would affect the blood sugar of cattle. This dose was very much larger than expected; in fact much out of proportion to the size of the animal. From 500 to 1200 units at one time seemed to be the amount necessary to produce the effects desired. The constituents of the blood and the milk were determined in the same manner as indicated in the preceding paper.

The Data

It was first thought by the writers that insulin could produce its effect in extreme form only in the presence of complete inanition. For that reason the first three cows treated were given only water. For the fourth cow, No. 47, no change whatever was made in her diet; this cow receiving her ordinary ration plus all the water that she wished.

The milk yield, the composition of the milk, and blood are all plotted against time and appear in Figs. 1, 2, 3, and 4. The units of insulin introduced into the blood are indicated by the dotted lines on the chart. The position of the vertical line represents the time at which the insulin was introduced into the jugular vein.

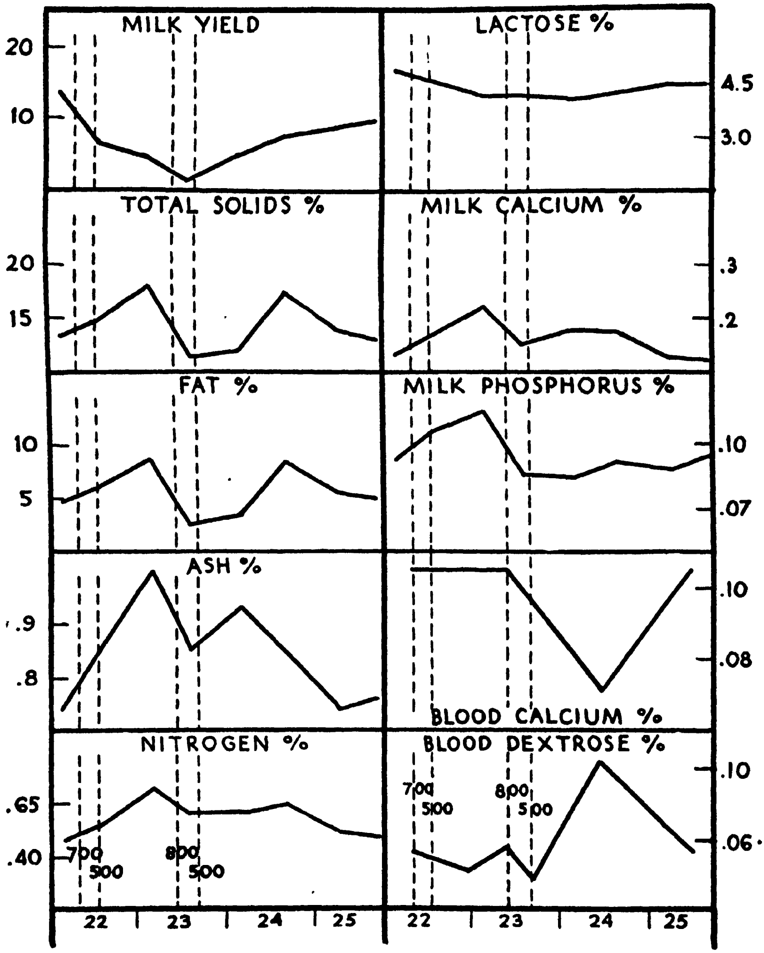

Fig. 1. Cow 47, the effect of insulin on milk secretion. Insulin treatment with full ration throughout is represented by the solid line.

The immediate effect due to the introduction of insulin into the blood stream of the cow is to cause a sharp drop in milk production. This drop is more pronounced than is the drop for starvation. It

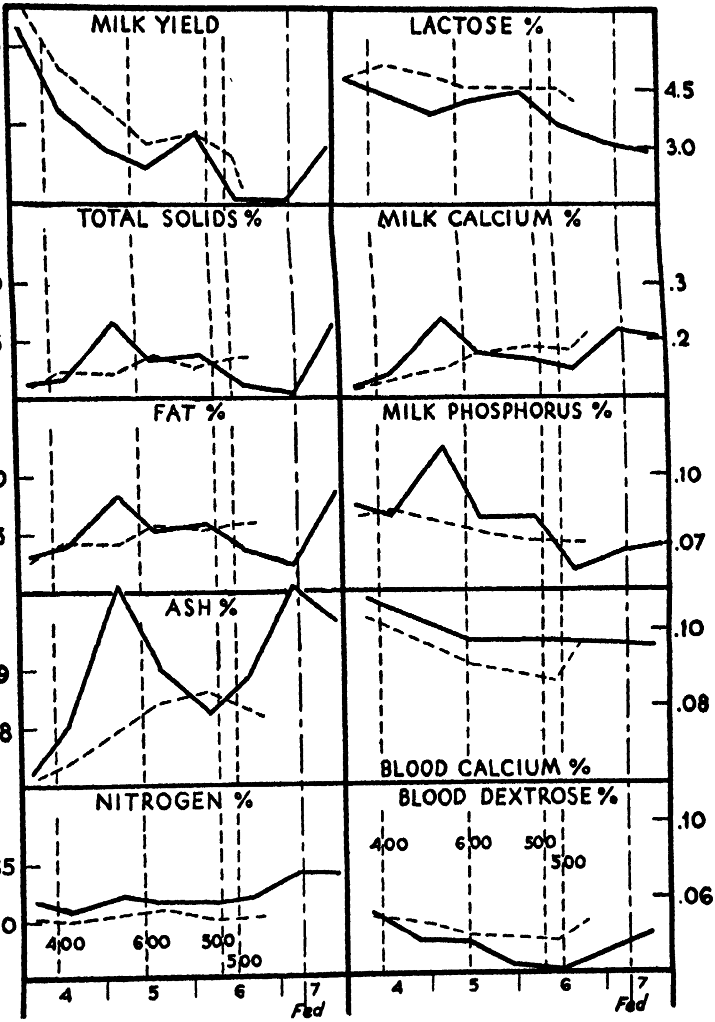

FIG. 2. Cow 52, the effect of insulin on milk secretion. Insulin with starvation is represented by the solid line; the dotted line shows the same cow with starvation only.

occurs whether the cow is kept under starvation or allo normal ration. The effect of the insulin treatment wore off m rapidly when Cow 47 was fed during treatment than when on s n. The effect of the insulin on Cow 141 in her second period was much more

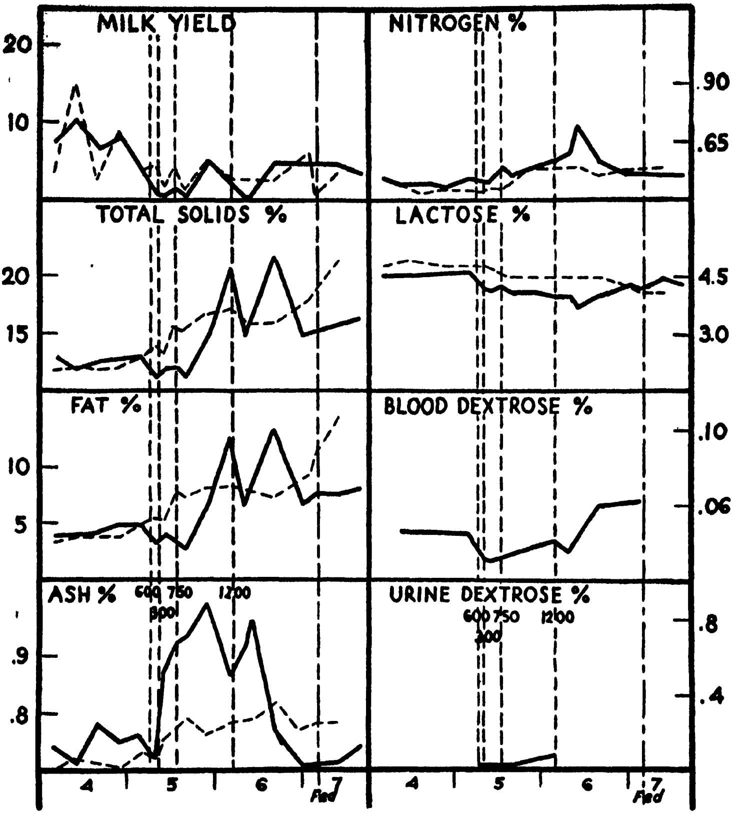

FIG. 3. Cow 141, the effect of insulin on milk secretion. Insulin with starvation is represented by the solid line; the dotted line shows the same cow with starvation only. The amount and time of insulin injections are shown by the vertical dashed line.

severe than it was for the other animals, as noted by the fact that she remained in coma for nearly 48 hours even though fed large quantities of dextrose and cane sugar. This severe reaction influenced the rapidity of her recovery in a marked degree, as noted by the length of time it took her to return to normal production and the changes observed in the composition of her milk during this period.

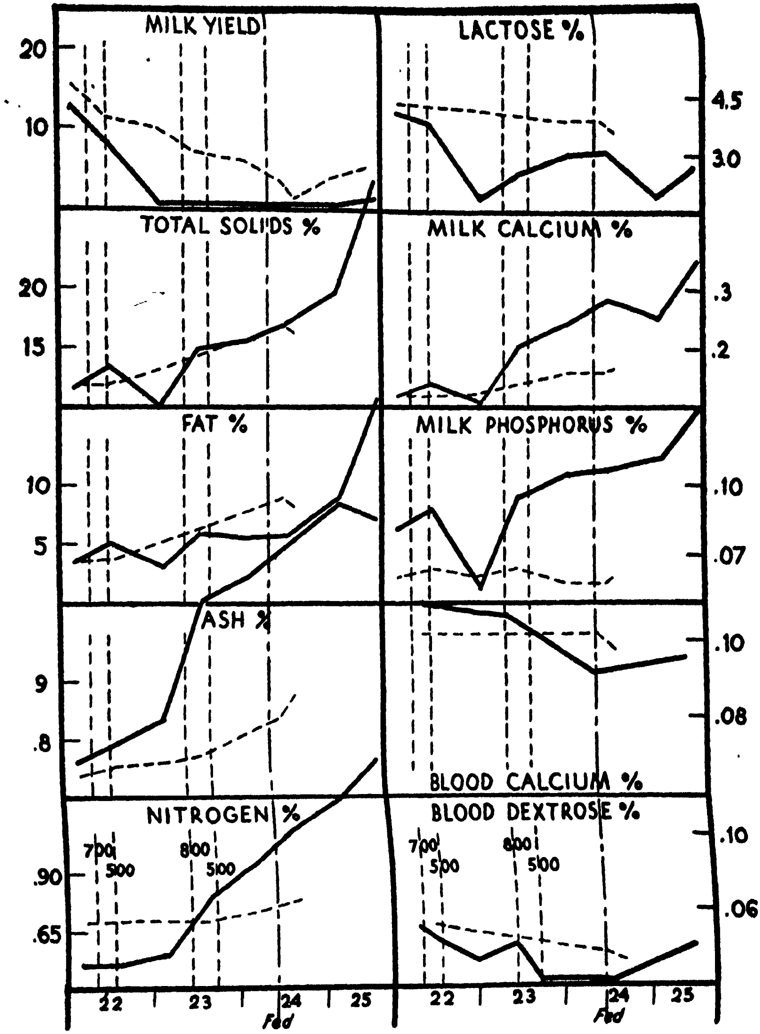

FIG. 4. Cow 141, the effect of insulin on milk secretion. Insulin with starvation is represented by the solid line; the dotted line shows the same cow with starvation only.

The total solids of the milk rose sharply after the introduction of insulin. The highest point reached by this rise in total solids was much greater than that noted in starvation for the same period; in fact the effects 16 hours after the introduction of insulin correspond to those found where the starvation has endured for 48 hours. From this high point the total solids tend to decline; the decline being most rapid in the cow which was fed. With the subsequent injection of insulin the total solids rose somewhat but not to the same extent as noted in the first instance. Cow 141, on the second experiment where the effect was so long protracted, showed a tremendous but late rise in her milk total solids, which was maintained for a period of 2 or 3 days, when it declined. This late rise was apparently due to the insulin shock through which Cow 141 had passed and the attendant rather protracted inanition.

The changes in the butter fat percentages were equally acute. A rise in the butter fat percentage to a level which was normally reached by starvation in 2 days was reached by the cows on insulin treatment in 16 hours. From this point the butter fat percentage declined on the third injection of the insulin. The curves of the butter fat percentage are quite different in the insulin treated cows than those for starvation alone.

The percentage of ash rose very rapidly after the introduction of insulin. This rise was noted whether the cows were starved or fed. The point reached by the rise in 16 hours was as high as that found for starvation after a period of 48 hours. The height of the rise was somewhat affected by whether or not the cow was fed; the cow being fed not going as high as the cow which was under starvation. The high point of the ash percentage on the first rise was about 10 per cent above normal. On the subsequent introduction of the insulin a second rise took place although not to the same level as the first one.

The nitrogen content of the milk behaved irregularly. Cow 52 under complete starvation had a slight rise in the amount of nitrogen with the introduction of insulin. This rise was probably not significant. Cow 47, adequately fed, showed a marked rise in the nitrogen value, about 1.5 times normal, from which point it declined somewhat to reach normal at the end of 3 days. Cow 141 in the first experiment showed a slight rise in nitrogen per cent at first and a marked rise after the third injection of insulin. Cow 141 on the second experiment showed little change in the nitrogen content of her milk after the first two injections, but after the third the rise was marked and continued. This cow's whole chart is of sufficient interest to warrant a separate chart to show the full trend of the cow's production on a smaller scale (Fig. 5).

The changes in the lactose per cent were equally striking, but in the opposite direction from those above noted. The lactose dropped rapidly to values which were a tenth lower than those noted under starvation. From these values the lactose tended to return somewhat toward normal until a subsequent injection of insulin when the drop was continued. The drop for Cow 141 in the second experiment was very severe indeed going to a lactose value less than half that normally found. Cow 52 showed a very pronounced drop when the second group of insulin injections was made.

The percentages of calcium and phosphorus in the ash of the milk tended to follow those of the ash itself, indicating that the ash composition was probably not changed markedly but was simply increased in amount.

The blood sugar percentages furnished some measure of the insulin effect and also of the amount of dextrose which would be available to the udder for the organization of the lactose of milk. The introduction of insulin into the blood stream has the very rapid effect of lowering the blood sugar. These blood sugar values sometimes go to the very low value of 20 mg. per 100 cc. or less. For the amounts of insulin used it would seem that the period of time necessary to accomplish this result was more than half a day. On the introduction of a subsequent injection of insulin the blood sugar tends to fall again sometimes to a new low level. From this point, if the cow receives no further interference, she slowly builds up the blood sugar to a point which, if she is receiving an adequate ration, may go considerably above the normal.

Before discussing these results, it is of interest to look at the curve for Cow 141 on the second experiment over a longer period of time. This cow was given the largest injections of insulin and over a shorter time, plus starvation during the period. She was much more markedly affected by the insulin than was Cow 52 which received insulin over a longer period of time, and to only 80 per cent of the amount. Shortly after the third injection of 800 units of insulin Cow 141 became

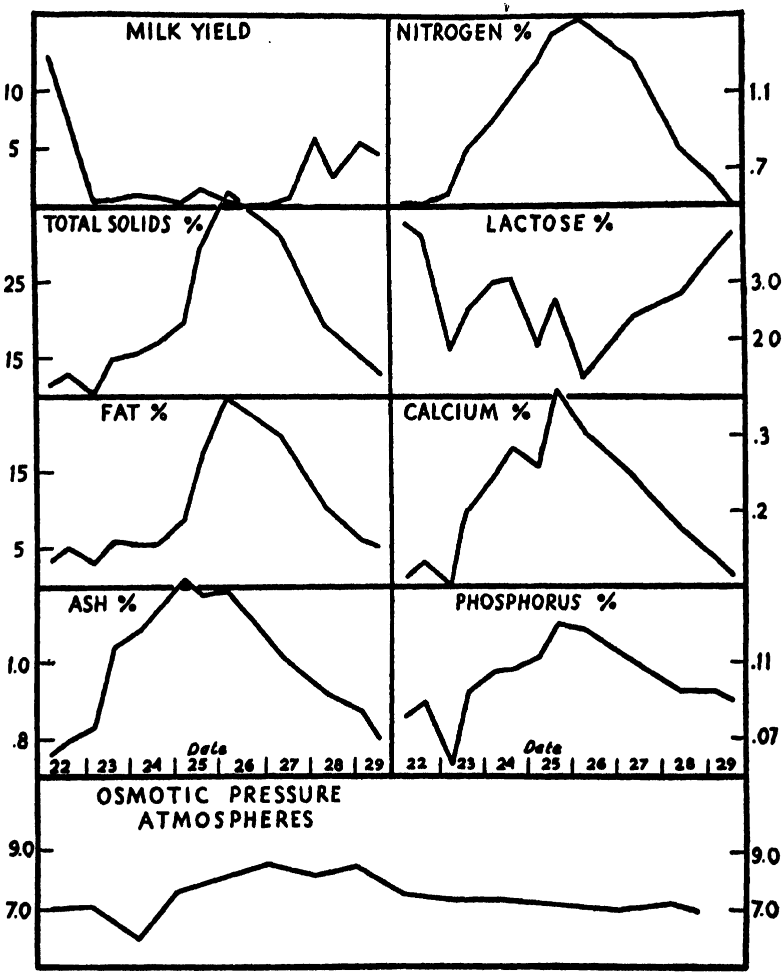

FIG. 5. Milk yield and composition of the milk of Cow 141 during insulin treatment and recovery.

partially paralyzed. After the fourth injection of 500 units the cow could no longer stand or use her legs but could only lie on her side. Her breathing was irregular; the limbs became cold. The cow then passed into coma and remained in this condition for about 12 hours when she was given an injection of 20 cc. of 10 per cent dextrose solution directly into her jugular vein. At the same time she was fed 500 gm. of dextrose as a water solution. In 20 minutes or so the cow was on her feet. But a short time after that she returned to her lying position and in spite of giving her further injections of dextrose and of feeding her large quantities of cane sugar no subsequent improvement took place for another 26 hours; a gradual improvement then commenced. At the end of about a week the cow's milk production returned to 75 per cent of her normal production and she was in other respects apparently well. The clinical aspects of this case clearly indicate that this cow was much more markedly affected than she was on her previous treatment the year before, or than was Cow 52 treated during the same time. This cow is, so far as the writers are aware, the only one exhibiting artificially produced insulin shock over a protracted period. The ability to produce such a prolonged shock, especially after the feeding and injection of dextrose, is therefore of much interest in view of the suggestion that milk fever is due to such a hypoglycemia of the blood brought on by a heavy milk secretion. The course of this cow's milk production and the composition of the milk in these periods are of significance to the study in hand. Fig. 5 gives the extended graph for this cow during the whole period but on a reduced scale.

The curve of milk production for Cow 141 over this extended period is characterized by a rapid drop in the quantity of milk produced after 16 hours from the time the first insulin was injected. This low production is continued over a period of 4 days, a length of time much longer than for the other cows which did not receive as severe a shock from the insulin. This is no doubt partly to be accounted for by the starvation attending the coma after the last injections of insulin. It is, however, to the insulin which must be attributed the majority of the effects since this cow was fed large quantities of sugar during this coma period. It is of interest to note that in spite of the severe effect of the insulin this cow returned to normal production after a time. The period is, however, much longer than for the cow fed during the insulin treatment, or for a cow given less insulin although starved during the period.

The highest total solids content of Cow 141's milk is reached at the relatively late period of 60 hours after the last insulin injection. The total solids content goes to a very high level resulting in a milk which is nearly solid. These levels are about three and one-half times the normal; the total solids content finally reaching 37 per cent. This rise is quite largely accounted for by the increase in the butter fat content of the milk from about 3.7 per cent to 24.8 per cent, or to 6.7 times the normal value.

The ash content likewise increases from 0.74 to 1.22 per cent, an increase of roughly 60 per cent. The curve for percentage of ash, while it rises, does not exactly parallel that for the butter fat percentage, as it reaches its crest at an earlier period and remains fairly constant at this level for a much longer time.

The nitrogen content of the milk likewise increases to a relatively high level. This increase is of little significance until the third injection of insulin when the rise commences. The high point is reached at a later period than that for the ash but at an earlier period than that for the butter fat percentage. The amount of nitrogen contained in the milk at that period is about three times as much as that ordinarily found. From this high point the nitrogen drops off and by the end of the experiment has very nearly reached normal.

The lactose percentages behaved irregularly but are throughout inverse to those of the other constituents of the milk. Within 16 hours after the injection of the first insulin the lactose of the milk has reached very low values. From this low point of 1.8 per cent there is a slight recovery until the effect of the second insulin injection is noted when the lactose returns to its low values. The lactose keeps these low values as long as the ash percentages remain high but when the ash percentages start to drop the lactose returns to normal. At the end of the experiment it is within 0.6 per cent of the normal value for this cow.

The calcium content of the ash follows the changes of the ash in large measure. It does, however, reach relatively higher values being at the crest of the curve about three times its normal value, whereas for the ash it is only 1.6 times the normal value. This possibly may be accounted for by the protein increase and the affinity of casein for calcium. The calcium content has returned to nearly normal values by the end of the experiment. The high point of the calcium corresponds exactly with the high point of the nitrogen.

The phosphorus content of the milk parallels almost exactly the calcium of the milk. Unfortunately the blood sugar and calcium were not followed further than the period noted on the earlier chart.

These studies show that the effects of insulin on milk secretion are much more severe than those of starvation in that the response is more immediate. They are characterized by a rapid drop in milk production, an increase in the total solids, butter fat percentage, ash, and to a limited degree in nitrogen. The changes in ash content appear to be fairly uniform for its constituents as the calcium and phosphorus very largely parallel the changes in the total ash. Lactose contrasts with the other milk constituents in that it rapidly declines in amount. It is also to be noted that the butter fat percentage does not appear to take the same course as that for the starvation results. The increase for insulin being almost immediate and then held at that level rather than a continuous increase over the period. Insulin results in a marked decrease in the dextrose content of the blood. The fact that this decrease in dextrose content is accompanied by a marked decrease in the lactose content of milk furnishes another independent link in the proof that lactose of milk comes from dextrose of blood.

As indicated for the starvation experiments, if the osmotic pressure of the milk is to remain constant in view of the decrease in lactose the salt content of this milk must increase rapidly. This is what actually happened. It is of interest to see how nearly alike the osmotic pressures of different periods in the experiment may be. Fig. 6 shows the graph for the osmotic pressures of the milk for the duration of the experiment.

Cow 47 on insulin treatment and full ration showed a slight increase in osmotic pressure for the third injection of insulin. The osmotic pressure after this point, however, returned below normal and remained essentially normal during the entire experiment.

Cow 52 on insulin treatment and starvation showed a like increase in osmotic pressure by the third injection. From this point, however, the milk returned to normal osmotic pressure and remained so throughout the experiment.

For Cow 141, despite the extreme variation which was produced in her milk, the osmotic pressure of this milk increased only a small relative amount; the increase being at maximum about 1/5 above normal.

In view of the fact that starvation increases the blood's osmotic pressure to some degree, the increase in the milk's osmotic pressure was to be expected rather than otherwise. The results of the experiments on insulin are in agreement with those on inanition in showing that the osmotic pressure of the blood is a leading factor in stabilizing the concentration of the solids found in milk.

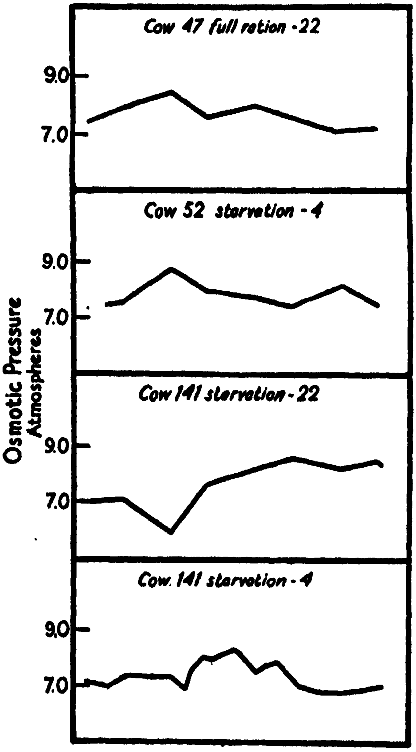

FIG. 6. Calculated osmotic pressures of cow's milk during insulin treatment.

If the trend of the butter fat percentages be compared with the trend of the other constituents in the milk, it will be noted that, as in the case of the starvation experiments, the percentages of butter fat are throughout somewhat independent of the other constituents. The results are consequently in accord with what would be expected from the previous work.

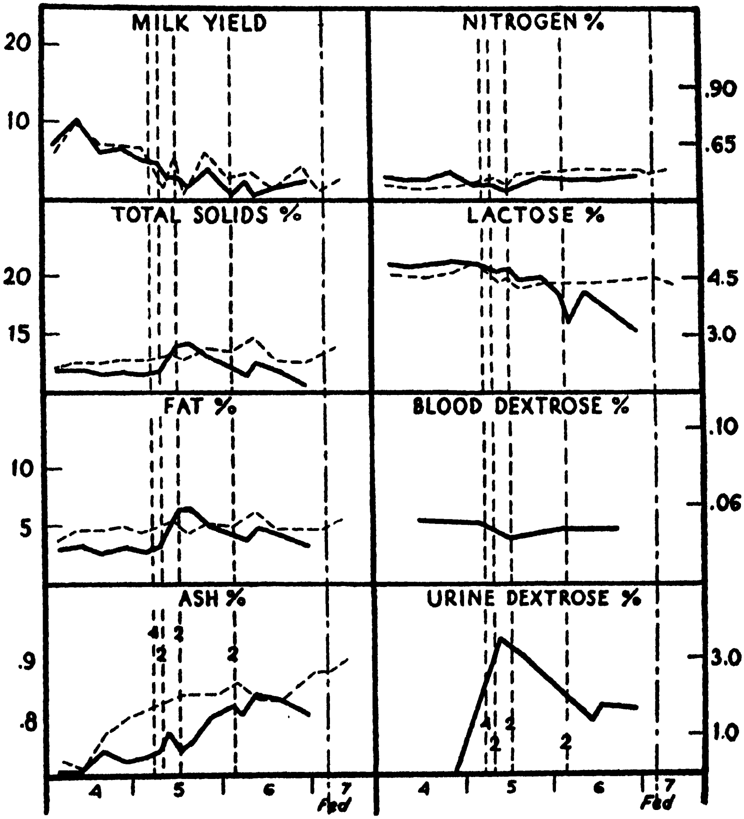

FIG. 7. Cow 47, phloridzin and complete inanition. Vertical lines on the chart show the amount and time of phloridzin injection. The solid line gives these results,—the dotted line gives the data on starvation alone.

Phloridzin

Since phloridzin has the property of causing the animal to excrete large quantities of sugar and nitrogen in the urine, and as a consequence to deplete the blood sugar after a time, it would seem as if its action should result in raising the ash content of milk and in decreasing the lactose content of the same when the cow has been injected with phloridzin, accompanied by starvation. The course of milk secretion with phloridzin would therefore be expected to be quite different than that for insulin. The salt-lactose balance should tend to remain constant, however, if the hypotheses developed in the preceding sections are correct.

Cow 47 was given over to this experiment. This cow was kept under the conditions of complete inanition. The phloridzin was introduced into the jugular vein as a 25 per cent alcoholic solution of the number of gm. indicated on the chart. The time of introduction of the phloridzin is indicated by the vertical dotted line.

As Paton and Cathcart and others (20) have shown for goats, the milk yield of this phloridzin treated cow when accompanied by starvation decreases in amount. The chart shows, however, that this decrease in amount follows essentially the curve of starvation and does not appear to represent any particular effect of the phloridzin.

The total solids of the milk increased somewhat immediately after the introduction of phloridzin, but then subsequently decreased to increase again after the fourth introduction.

The butter fat percentage follows a curve almost identical with that of the total solids. It is to be noted that the butter fat percentage goes somewhat above the starvation curve whereas the section on insulin accompanied by starvation showed that the butter fat percentage was reduced somewhat.

The ash content of the milk rises in a manner comparable with that occurring in starvation but at a slightly lower rate. It meets the starvation curve, however, at the end of the experiment. This fact is of interest since it is opposite to that noted when insulin is introduced into the blood stream.

The nitrogen content of the milk started higher than it did for the same cow under the straight starvation experiment but after the introduction of phloridzin it dropped to a lower level than that noted for the starvation experiment, and remained slightly lower, although paralleling the starvation results, for the rest of the period.

The results for the lactose are much the same as those when the cow is under simple starvation save for the fact that the drop in lactose is very marked at the end of the experiment when the reserved body sugar has been depleted. This result is different from that observed in the insulin experiment and as noted earlier is to be expected from the difference in action of the drugs.

The tests on the blood sugar show that phloridzin depleted the blood sugar to a limited degree but that the cow recovered her normal blood sugar in a relatively short period after the introduction of the phloridizin. The degree of depletion through the action of phloridzin is not as pronounced as that noted for insulin.

The urine nitrogen and the urine dextrose were markedly increased by the introduction of the phloridzin as has been noted in all corresponding experiments.

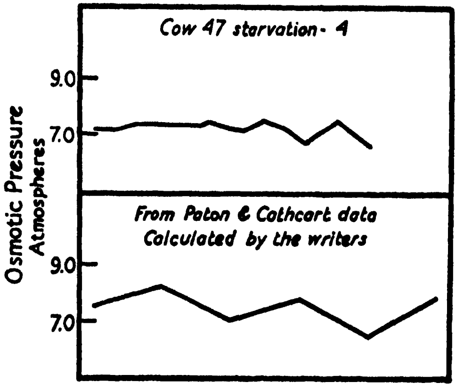

FIG. 8. Calculated osmotic pressures of cow's and goat's milk when under phloridzin treatment.

So far as milk secretion is concerned the results are an increase in the fat and ash content of the milk, a decrease in the lactose percentage and a decrease in the total milk flow. The depressed dextrose of the blood without the corresponding changes in the blood salts, save for a slight depression of the potassium in phloridzin diabetes, will account for the observed results in the milk secreted.

The approximate calculated osmotic pressure of this milk and of the goat's milk in the experiments of Paton and Cathcart are presented in Fig. 8. These osmotic pressures are found to be constant throughout the duration of the experiment indicating again the balanced condition between the salts and sugars of blood and milk in milk secretion.

DISCUSSION AND SUMMARY

The four types of experiments on milk secretion herein described really fall into one general class so far as the physiological effects produced are concerned. Starvation lowers the blood sugar and raises the osmotic pressure of the blood. The experiment using parathyroid hormone with or without starvation may have its effects interpreted as simply due to starvation since 1000 units of this hormone produced no visible effects on the blood calcium or milk constituents different from those of starvation. Since insulin produces a marked and rapid drop in blood sugar it too may be looked upon as a rapid starvation effect. It has some other important effects, however. Briggs *et al.* (21) have shown that potassium and phosphorus of the blood are decreased and Luck, Morrison, and Wilbur (22) indicate a reduction in the amino acids of the blood in insulin treatment. Phloridzin lowers the threshold for sugar retention with the consequence that in time it tends to lower the sugar of the blood to an even greater extent than that noted in starvation. It tends to depress the potassium, to increase the phosphorus content of the blood, and to cause the body to burn protein rather than carbohydrate, thus increasing nitrogen excretion.

All of the experiments are characterized by a sharp reduction in the milk yield. Cary and Meigs (23) have studied like reductions in milk yield produced by varying the energy or protein of the diet. They conclude that such decrease in milk production may be interpreted as due to the direct effect of the starvation and the consequent reduction of the energy and protein available to milk secretion.

The reduction in milk yield for the experiments herein described can undoubtedly be attributed to the same causes as those cited by Cary and Meigs. The experiment where Cow 47 was given a full ration and at the same time injected with large quantities of insulin is of particular interest in this connection. The ration was adequate and the cow ate well, yet her production declined to a fifth of her normal milk yield. Her chart shows that there was a slight reduction in her blood sugar when insulin was introduced into the blood stream. It seems furthermore likely that this sugar was not as available to milk secretion, since there appears to be more than a corresponding drop in the lactose content of the milk. The work of Luck *et al.* would seem to indicate that there should be a like drop in the amino acids of the blood. These two conditions would lead, according to the work of Cary and Meigs, to a reduction in the concentration of the nitrogen of the milk. Actually, in the experiment as it was performed, the nitrogen increased to a value about 40 per cent above normal. A somewhat similar conflict is noted in two of the other three insulin experiments where starvation accompanied insulin injection. To this extent it would seem that the factor deserving most emphasis in its immediate effect on milk yield is the energy available, and that the later and more secondary factor is the amino acid concentration of the blood.

In the starvation experiments, the butter fat percentage of the milk rises rather uniformly with the duration of starvation. In the insulin experiments, however, the charts appear to show a marked reduction in this butter fat percentage immediately after the introduction of insulin. This is particularly noticed after the second and third injections. Since the dextrose of the blood tends to be reduced and made unavailable to the general physiological processes by the presence of the large excess of insulin, and since this reduction of the butter fat percentage is noted as an accompanying phenomenon, it would appear that the blood dextrose plays a part in the synthesis of milk fat as well as being the source of the milk lactose, possibly as a source of energy in converting body fat to butter fat. In this regard the results for the treatment of Cow 47 with phloridzin are of importance. As noted by others, the introduction of phloridzin causes a marked rise in the fat percentage of the milk. The lactose per cent is also higher than that noted in starvation. Since phloridzin, by lowering the threshold for the blood sugar, causes large quantities of it to be drained from the body through the urine, and therefore reduces the reserve supply, it follows that if the insulin hypotheses are correct we should expect an eventual lowering of the lactose and of the fat below the starvation level. During the last of the experiment this is what was actually observed.

The effects of starvation and of insulin furnish concordant proof for the theory that the lactose of milk is derived from the sugar of the blood.

The fact that the different constituents of the milk, the fat, the lactose, the nitrogen, and the ash, do not exactly parallel each other in their behavior throughout these experiments indicates that they have in all probability separate origin. This is particularly true of the butter fat percentage, which appears to have a rate of secretion which is more or less independent of the other constituents, and higher in amount. This result would fall in line with the conclusion of the writers in a previous paper in which it was indicated that the fat of the blood was very likely deposited in the udder as fat corresponding to body fat from which source it was metabolized into the fat of milk shortly before it was needed for milk secretion.

The wide variation brought about in the constituents of the milk by the treatment all point to the conclusion that in milk secretion a balance is maintained between the osmotic pressure of the milk and of the blood. Thus when the sugar of the milk is reduced either through starvation or by insulin the ash constituents rise to compensate for this reduction and make the osmotic pressure of the milk similar to that of the blood. These results further appear to indicate that the salts and the sugars are more or less independent in their passage and metabolism into milk from the other constituents. These observations are therefore in line with those obtained by Jackson and Rothera (14) and by Davidson (15) in their brilliant experiments where they modified milk secretion by returning milk or milk sugars and salts to the udder.

These experiments give direct proof for the conclusion that modifications of the blood of dairy cattle produce direct and predictable modification of the milk secreted.

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BODY PATTERN AS RELATED TO MAMMARY GLAND SECRETION

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(Communicated August 13, 1931)

The effects on body form of the testis as observed in castration—or of the pituitary as noted when it is pathological or experimentally removed as in the experiments of Cushing, Smith, and others, or altered by the animal's heredity as observed in the experiments of MacDowell and Smith—or of the ovary as seen in the work of Goodale on the plumage and structure of birds have made it good form to think of body form as a function of gland secretion rather than gland secretion as a function of body form. The case is reversed, however, when the dairy cattle breeder thinks of his cattle. LeCouteur's paper called attention to this relation of body pattern to milk secretion about 1834. Since that time the relations between body form and milk secretion have grown to include many more points of the growth pattern until more than twenty separate items of the body structure are included in those supposed to affect the amount of milk the cow is able to secrete.[1] Examination of the literature on the subject, however, reveals the fact that in a scientific sense the proof for these supposed relations is largely lacking.

In 1914 the writer commenced a study which has grown to include numerous aspects of this problem. The latest research has about 6000 Jersey cows measured in eight different particulars for its basic data, the measurements being collected by the American Jersey Cattle Club. These measurements include weight, height at withers, depth at withers, heart girth, paunch girth, width at hips, body length, and rump length. Besides these data, the age and parentage of all cows were known from their registration papers. The milk productions and butter-fat yields, etc., were known on about 1500 with Registry of Merit records. The bearing of these records on the problem of body pattern as related to mammary gland secretion is presented in this paper.

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[1] Humphreys, G. C., "Conformation in Relation to Production," World's Dairy Congress, pp. 1362–1375, 1922.

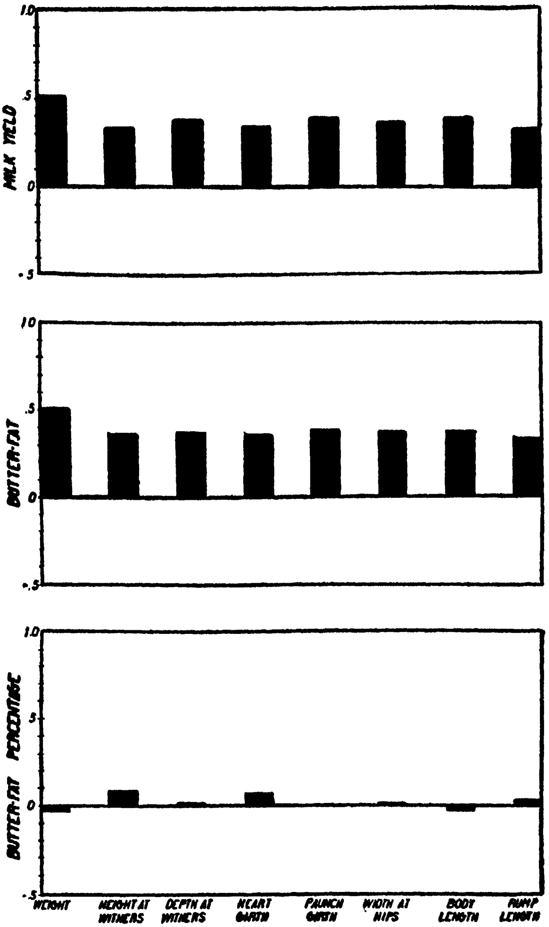

FIG. 1. Relation between conformation and production of Jersey Registry of Merit cows, all ages.

To fully appreciate this problem it is necessary to approach it in the same manner as the dairyman has done. In the early days little was realized as to the effects of the age of the cow on her milk production and the correlations thus developed between her body form and milk production. These correlations between age and milk yield or body size are of the order of 0.5. Correlation of such magnitude cannot, of course, be neglected in any true analysis of the problem.

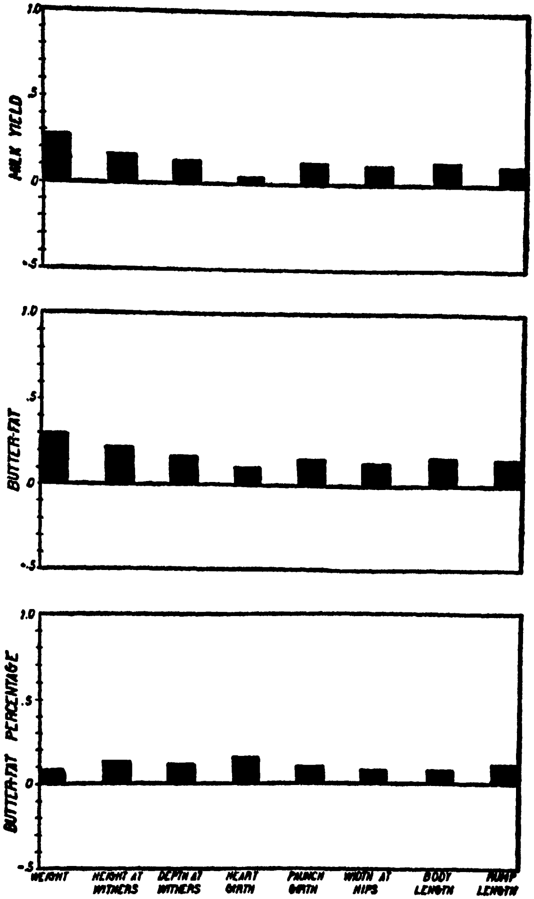

FIG. 2. Relation between body pattern and mammary gland secretion, influence of age removed by converting all records to their equivalent at the age of 8 years and 3 months.

However, they were and it remains for us to show their bearing on the observed relations between body pattern and milk secretion. Figure 1 presents the correlation between body size and milk secretion, butter-fat yields, and butter-fat percentage without regard to age. The height of the bars represent value of the correlation coefficients.

The marked influence of body pattern on the quantity of milk secreted and its almost complete lack of effect on the proportion of the constituents within this milk is the most striking feature of data of figure 1. The next most noticeable conclusion is the preponderance of the influence of weight or mass of the cow on the quantity of milk she is able to secrete as contrasted with the other elements of type. The other measurements of the body pattern appear to have equal influence on the quantity of milk which the cow is able to secrete. These correlations are materially influenced by the age of the cow, however. This age effect is removed in the data of figure 2 by correcting each measurement to its corresponding size at the age of 8 years and 3 months.

Comparison of the data of figure 2 with those of figure 1 shows the correlations are reduced to half their previous values by taking into account the influence of age on body size and milk secretion. Age is thus a very important variable in the relation of body form and mammary gland secretion. The next fact to strike the eye is the relation which weight bears to milk secretion as contrasted with that which the bone measurements bear to milk secretion. Weight is considerably more important. This is apparently due to the fact that weight measures not only the skeletal size but also the fleshing which the animal carries on this skeleton.

The next step in the analysis of this problem calls for the determination of the relation between the amount of milk secreted as influenced by the variation of the eight different measurements of body pattern as they separately affect the amount of this secretion. The partial correlation coefficients for milk yield with one of the items, the rest being held constant, furnish a measure of this relation. The correlation coefficients are shown in figure 3.

The data of figure 3, in the light of those in the preceding figures, show that the age of the animal, her weight, and her heart girth are in the order named of significance to milk secretion. The other six measurements of body pattern have insignificant effects. The influence of age on milk secretion has been repeatedly discussed and needs no further amplification. The weight of the cow for her age is of those studied the most important single measurement of body pattern. The

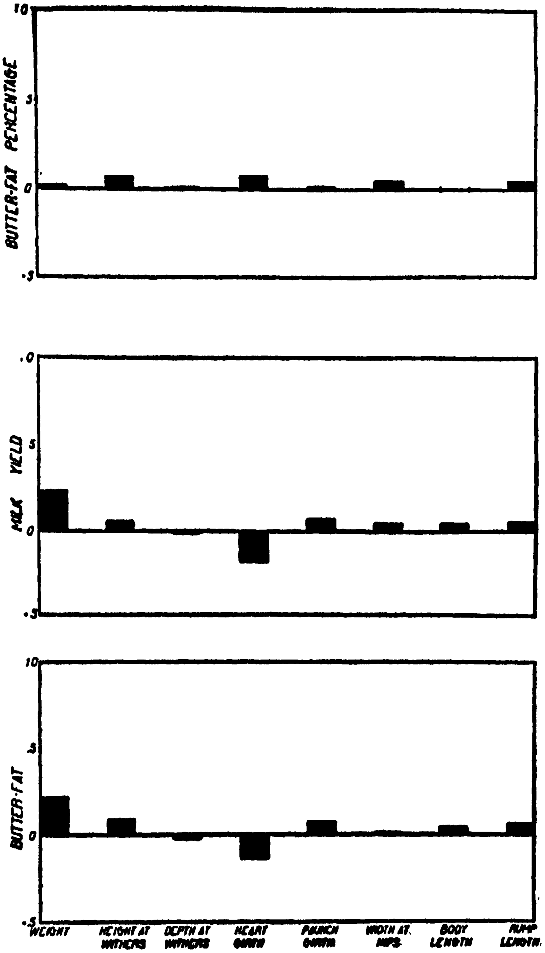

FIG. 3. Partial correlations between body pattern and mammary gland secretion. Throughout the effect of the given body part on milk secretion as measured is distinct from any influence of the other parts. In all cases age as well as the other seven variables is held constant.

larger the cow the larger her milk yield. Weight measures both the degree of fleshing and the general skeletal structure. It follows therefore that both of these items are important to milk secretion. The data are not such as to allow us to discuss the problem of the economics of this production either from the point of view of the protein and calories consumed and returned or the cost in dollars spent and returned. The third significant point is that a definite body pattern favors the secretion of a large quantity of milk. The wedged-shaped appearance of the cow at the shoulders when viewed from the front favors higher milk production than a heavy rounded conformation. This follows from the correlations showing that the cow of relatively small heart girth tends to have the larger milk yield and that the depth of this girth play has little or no part in milk secretion. The last fact coming from these correlations is the uniform relative unimportance of the other eight points of body pattern.

The negative side of the evidence is of equal interest. It is asserted that the wedge—observable by viewing the cow on the side—bounded by the back line, the hip height, and the lower line of the body is directly indicative of the amount of milk secreted. The fact that the measurements of depth at withers, paunch girth and body length show no marked association with the amount of milk secreted casts grave doubt on this assertion. The fact that the length of rump fails to show any important relation to the amount of milk secreted leads to an adverse conclusion to the oft-repeated assertion that the length of the rump limits the length of the udder and by so doing limits the milk secreted. This feeling of distrust is further borne out by the fact that the width at the hips, another supposed measure of udder size, also shows no important relation to the cow's milk yield. The width at the hips and the body length are functions of the third of the so-called three body wedges which are supposed to be indicative of milk secretion. This wedge is observed in looking down upon the cow. As neither of these measurements has any important association with milk secretion it follows that this wedge has but little significance. Only the quantity of milk secreted or the quantity of the butter-fat are affected by the body pattern. The butter-fat percentage has throughout only insignificant correlations to the body measurements.

The data herein presented allow of the conclusion that two items of body pattern, weight and a wedged-shaped form when viewed from the front are to a limited degree indicative of the amount of milk the cow will secrete. Quantitatively these correlations have a significance comparable to those respectively for the milk yields between half-sisters and single cousins. The other items studied are without significance. None of the items were indicative of the concentration of the butter-fat in the milk.

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